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Fate and effects of two pesticide formulations in the invertebrate *Folsomia candida* using a natural agricultural soil

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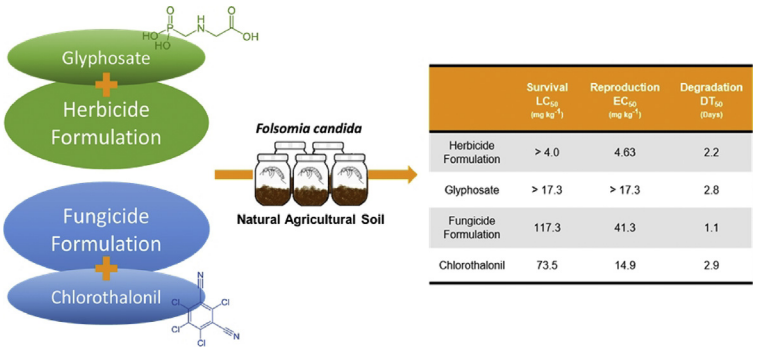
HIGHLIGHTS

- Glyphosate and chlorothalonil formulations/active ingredients were studied.
- The tested chemicals rapidly degraded in a natural agricultural soil.
- Sub- and lethal toxicity of chlorothalonil largely depended on its chemical formulation.
- Sensitivity of springtails was pesticide-specific.
- Chlorothalonil was the most toxic chemical to *Folsomia candida*.

GRAPHICAL ABSTRACT



	Survival LC_{50} (mg kg^{-1})	Reproduction EC_{50} (mg kg^{-1})	Degradation DT_{50} (Days)
Herbicide Formulation	> 4.0	4.63	2.2
Glyphosate	> 17.3	> 17.3	2.8
Fungicide Formulation	117.3	41.3	1.1
Chlorothalonil	73.5	14.9	2.9

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ABSTRACT

Degradation rates of two widely used pesticides were assessed, and acute and chronic effects on a standard invertebrate species investigated. An herbicide (Montana®) and fungicide (Bravo500®) formulations were investigated and results were compared to the isolated active substances of each formulation (glyphosate and chlorothalonil, respectively). Tests were performed using the invertebrate *Folsomia candida* as test species and an agricultural natural soil. Degradation rate tests were determined under aerobic conditions at 20 ± 2 °C, using an ecologically relevant concentration of 5 mg (a.i.) kg^{-1} of soil for both chemicals. Results demonstrated degradation half-lives (DT50) of 2.2 days for Montana® and 2.8 days when pure glyphosate was tested. Values of 1.1 and 2.9 days were registered for Bravo500® and its active substance chlorothalonil, respectively. There were no effects on survival for the tested concentrations of both forms of the herbicide (up to 17.3 mg kg^{-1}). However, reproduction was affected, but only by the herbicide formulation, with an estimated EC50 value of 4.63 mg (a.i.) kg^{-1}. Effects were most unlikely related to glyphosate. For chlorothalonil, both tested forms affected survival and reproduction. The estimated LC50 values were 117 mg (a.i.) kg^{-1} and 73.5 mg (a.i.) kg^{-1}, and the EC50 41.3 mg (a.i.) kg^{-1} and 14.9 mg kg^{-1} for the formulation and the active ingredient, respectively. The effects of the active

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ingredient were significantly stronger, indicating the major influence of the active substance in the effects caused also by the formulation. Overall results demonstrate the importance of evaluating the effects of the formulated chemicals, as they are applied in the field, and not only their isolated active ingredients.

1. Introduction

Most ecotoxicological tests are performed under controlled conditions in the laboratory, essentially because field testing may experience high variability, often hampering results interpretation (Römbke et al., 2006). The developed standardized guidelines used to perform such soil studies became reference procedures over the last 30 years (Jänsch et al., 2005). For this purpose, reference soil substrates (e.g. LUFA natural soils; artificial standard soils) were developed by standardization authorities and have been used in most ecotoxicological analyses allowing comparability of results. However, it is difficult to extrapolate the results of reference soil tests (whether natural or artificial) to field situations where soil properties might be completely different (Römbke et al., 2006). The use of natural site soils may then be more ecologically relevant and representative of the conditions from a particular area/region (Leitão et al., 2014). In an attempt to bring more "realism" to these evaluations, the number of reports giving more relevance to natural agricultural soils instead of the standardized soils in soil contamination studies is increasing (Okada et al., 2017; Santos et al., 2012; Silva et al., 2017). Since each specific geographic location presents different soil features, there is the need to gather realistic and integrated information from different natural soil substrates upon contamination.

Synthetic chemicals (e.g. pesticides) usage has increased drastically in the past decades in agriculture. Glyphosate (Gly; CAS 1071-83-6) is the active ingredient (a.i.) of the most intensively used herbicides around the world to control weed development and protect crops, with several formulations available in the market. This is a very efficient systemic herbicide that acts by blocking the plant's shikimic acid pathway, which is not found in animal cells (Von Mérey et al., 2016). However, there is extensive literature on this systemic herbicide, testing the chemical alone or as commercial formulations and reporting harmful effects in different environmental compartments, while also posing risks to humans (Myers et al., 2016). Despite the controversy on the use of glyphosate-based herbicides in the European Union (EU), the European Commission recently renewed the approval of glyphosate for a period of 5 years, after the scientific opinion given in 2017 by European Chemical Agency (ECHA) on the classification of glyphosate.

Fungicides are also an emerging chemical class of concern, receiving much less attention than well-studied chemicals such as herbicides or insecticides (Elskus, 2012). Chlorothalonil (tetrachloroisophthalonitrile; CAS 1897-45-6) is the active ingredient of non-systemic fungicides with very effective agricultural usage around the world, due to its reported multi-site contact-activity mode of action. Its fungal primary toxic mode of action occurs through binding glutathione, inactivating glutathione dependent molecules that are generally involved in detoxification, antioxidant protection against reactive oxygen production, and cellular respiration (Cox, 1997; Elskus, 2012). The chlorothalonil-based fungicides are therefore highly efficient and consequently, widely commercialized (Cox, 1997; Zhang et al., 2016). Industrial use of chlorothalonil has also experienced recent applications as a preservative of paints, resins, emulsions, among others (Cooke et al., 2017), facts that have contributed to its environmental dispersion.

Overall, pesticides are commercialized with other ingredients (often considered as "inert" substances) incorporated in a final formulation. These substances are added to enhance stability and action of the active substances, but their influence in the overall activity is often unknown (Mesnage et al., 2015). It is therefore important to evaluate the role of the active ingredient (a.i.) in the formulation but also the overall effects of the formulation in soil biota and also its chemical properties (e.g. degradation rates in soil), after application. Soil biota play an important role in soil functioning, also providing a practical tool for assessing soil quality status (Briones, 2014). The arthropod *Folsomia candida* (Willem 1902) is one of the most used species in ecotoxicological testing since it has a key position in the soil food web as a prey and consumer (Fountain and Hopkin, 2005), being also an ecotoxicological model organism relatively easy to be kept in the laboratory.

This study was outlined to characterize the fate and effects of two widely used pesticides, using *Folsomia candida* as a representative of non-target organisms. Comparative adverse effects between formulated and isolated active ingredients were evaluated, using a natural agricultural Mediterranean soil, and their persistence in soil was evaluated through degradation tests under controlled conditions.

2. Materials and methods

2.1. Test soil, pesticides, and organisms

The test soil was collected from an agricultural area from the lower Mondego valley in Coimbra, Portugal (40°12′44.0″N; 8°27′02.4″W). The area was kept in fallow, without use of pesticides, for over 10 years. Prior to soil collection, plant cover was cut and the soil tilled. Around 300 kg of soil were collected, sieved (5 mm), and defaunated using two alternating freezing-thawing cycles (24 h at −20 °C followed by 24 h at 20 °C). Soil was then kept at 4 °C until use. The physicochemical and mineralogical characterization of the natural soil was performed in Eurofins GmbH (Jena, Germany), an accredited test laboratory, and details are provided in Table 1.

Glyphosate-based herbicide (Montana®) was acquired from SAPEC, Portugal, with glyphosate as a.i. (30.8%, w/w) in the form of isopropyl ammonium salt ($C_6H_{17}N_2O_5P$) and a polyethoxylated tallow amine adjuvant (POEA; CAS 61791-26-2), with concentrations ranging from 7 to 12% (w/w). The fungicide formulation (Bravo500®) was acquired from Syngenta, Portugal, with chlorothalonil as a.i. (40%, w/w). Pure analytical compounds (glyphosate and chlorothalonil) were acquired from Sigma-Aldrich (Steinheim, Germany).

The collembolan *Folsomia candida* was used as test organism in laboratory reproduction tests. The test organisms were obtained from cultures in the Soil Ecology and Ecotoxicology Laboratory of the Centre for Functional Ecology, University of Coimbra, Portugal. The animals were maintained in vessels with the bottom filled with a wet mixture of plaster of Paris and activated charcoal (11:1, w/w) in a climatic chamber at constant temperature of 20 ± 2 °C, and with a 16:8 h light:dark photoperiod, and fed with baker's yeast (Vahiné, Mccormick, Italy).

2.2. Soil contamination

All tests were performed in laboratory using the natural agricultural soil, spiked with pesticides and moistened to 50% of the water holding capacity (WHC). To estimate a 50% of inhibitory effect compared to control on reproduction (EC50) and survival (LC50), several dilutions of a stock solution for each pesticide were prepared in distilled water, except soils spiked with pure chlorothalonil active ingredient (a.i.) where stock solution and respective dilutions were prepared in acetone. For pure chlorothalonil a.i., an extra control treatment was included with a volume of acetone equivalent to the total volume of spiking solutions used in the other treatments of this pesticide. Dilutions were

Table 1
Physicochemical and mineralogical characterization of the agricultural soil used in the present study, from the lower Mondego valley in Coimbra, Portugal. LOQ – limit of quantification; pF – soil moisture tension; wK – water capacity.

Parameter	LOQ	Value	Unit
Determination from the original sample			
Dry substance (105 °C) (EN 14346)	0.1	94.2	% w/w
Dry substance (105 °C) (EN 13040)	0.1	94.2	% w/w OS
pH Value (ISO 10390)		7.1	
Loss of ignition of the dry substance (EN 15169)	0.1	5.0	% w/w DS
Total nitrogen (ISO 13878)	0.05	0.20	% w/w DS
Bulk density (according to BGK e.V)	10	1300	g/l DS
Bulk density (according to BGK e.V)	10	1400	g/l OS
Sieve analysis >0.063 mm		68.8	% DS
Total sand (0.063 mm–2 mm) (ISO 11277)	1	62	% w/w ds
Total silt (0.002 mm–0.063 mm) (ISO 11277)	1	28	% w/w ds
Total clay (<0.002 mm) (ISO 11277)	1	10	% w/w ds
Maximum water capacity			
pF 1,8 (DIN 18121)		17.7	w%, w ($p_{1.8}$)
pF 1,8 (DIN 18121)		25.9	vol%, Θ ($p_{1.8}$)
wk max (DIN 18121)		30.0	w% w (p_m)
wk max (DIN 18121)		44.0	vol%, Θ (p_m)
Determination from the aqua regia digestion			
Lead (ISO 17294-2)	2	23	mg kg^{-1} DS
Cadmium (ISO 17294-2)	0.2	< 0.2	mg kg^{-1} DS
Calcium (ISO 17294-2)	20	5400	mg kg^{-1} DS
Total chromium (ISO 17294-2)	1	13	mg kg^{-1} DS
Iron (analog ISO 17294-2)	5	16,000	mg kg^{-1} DS
Potassium (ISO 17294-2)	5	1900	mg kg^{-1} DS
Cobalt (ISO 17294-2)	1	8	mg kg^{-1} DS
Copper (ISO 17294-2)	1	15	mg kg^{-1} DS
Magnesium (ISO 17294-2)	30	3600	mg kg^{-1} DS
Manganese (ISO 17294-2)	1	270	mg kg^{-1} DS
Sodium (ISO 17294-2)	10	110	mg kg^{-1} DS
Nickel (ISO 17294-2)	1	12	mg kg^{-1} DS
Phosphorus (ISO 17294-2)	10	461	mg kg^{-1} DS
Zinc (ISO 17294-2)	1	63	mg kg^{-1} DS

mixed into soil portions at the same day that the test started, except in soils used for tests with pure chlorothalonil a.i. These latter soils were prepared one day before the beginning of the test to allow acetone to evaporate overnight. Test concentrations of commercial formulations were defined according to nominal concentrations of the active ingredients. The nominal concentrations prepared for each pesticide are presented in Table 2.

2.3. Degradation tests

The degradation rates of the commercial formulations Montana® and Bravo 500® and respective pure a.i. (glyphosate and chlorothalonil) were characterized in laboratory degradation tests using spiked natural soils and by estimating the half-life (DT50) of each pesticide. The DT50 determinations were performed according to the OECD standard (OECD, 2009) and European Commission (EC, 2002) guidelines. Soil was previously spiked with nominal concentrations of the isolated active ingredients and in their respective formulations, to reach final concentrations of 5 mg (a.i.) kg^{-1}. These applications were made according to the expected concentration in soil after a single recommended dose application, which is 4.32 kg/ha for glyphosate (Von Mérey et al., 2016) and 3 kg/ha for chlorothalonil (Wu et al., 2012; Zhang et al., 2016). Pesticides were diluted in distilled water to moisten the soil to a level corresponding with 50% of the water holding capacity. Incubation experiments for each soil/pesticide combination were carried out on independent sampling vials, each containing 50 g of spiked soil. Soil samples were incubated at 20 °C with a photoperiod of 16:8 h (light:dark), with a weekly aeration. Samples were collected after 0, 1, 3, 7, 10, 15, 30, and 60 days (Montana® and glyphosate) and 0, 1, 3, 7, 10, 15, and 30 days (Bravo500® and chlorothalonil) of incubation, to measure the residual concentrations from three independent replicate vials.

2.4. Reproduction tests

The collembolan reproduction tests were performed following the procedures described in the ISO guideline 11267 (ISO, 1999). Synchronized organisms (10–12 days old) were exposed to the analytical concentrations of pesticides mentioned in Table 2, plus control, maintaining temperature and photoperiod at 20 ± 2 °C and 16:8 h (light:dark). Five replicates per treatment were used, each with 10 organisms in 30 g of soil (fresh weight equivalent; FW) in a 100 mL glass vial. Organisms were fed at the beginning of the test and after 14 days by the addition of 2 mg of dry yeast spread in crumbs on the surface of each replicate. Soil moisture content was maintained weekly by the addition of few drops of distilled water to reestablish the initial weight of each glass vial. All replicates were aerated weekly by opening the glass vials for few seconds. After 28 days, the content of each replicate was carefully transferred to small vessels, which were subsequently filled with water. After the addition of a few drops of blue ink and gentle stirring, the collembolans floating on the water surface were photographed and the number of juveniles and surviving adults was determined using image analysis software provided by ImageJ (version 1.49). Missing adult springtails were considered dead. An additional replicate per test concentration, but without collembolans, was prepared and submitted to the same conditions for pH and moisture measurements at the end of the test. This way, soil moisture content and pH were measured at the beginning and at the end of the test.

2.5. Pesticide residues in soil

Glyphosate analytical determinations followed methods described by the European Union Reference Laboratories, EURL (European Commission; www.eurl-pesticides.eu/). Moist soil subsamples of 100 mg were weighed into 15 mL sterile polypropylene centrifuge tubes and 1 mL of acidified water (formic acid to pH 2.5) was added to each tube. Samples were then placed in an ultrasonic bath with a frequency

Table 2
Nominal and measured concentrations (expressed in mg kg^{-1}), with the respective percentages of recovery (%), of glyphosate and chlorothalonil in formulations (Montana® and Bravo 500®, respectively) and as pure active substances in the spiked natural soils used in the laboratory reproduction tests with *Folsomia candida*.

	Montana®			Glyphosate			Bravo 500®			Chlorothalonil		
	Nominal	Analytic	%	Nominal	Analytic	%	Nominal	Analytic	%	Nominal	Analytic	%
C0	0	<0.05	0	0	<0.05	0	0	<0.05	0	0	<0.05	0
C1	0.135	0.15	111	0.27	<0.05	0	1.875	1.61	85.9	1.875	1.66	88.5
C2	0.27	0.19	70.4	0.54	1.38	256	3.75	2.82	75.2	3.75	2.58	68.8
C3	0.54	0.29	53.7	1.08	2.18	202	7.5	4.99	66.5	7.5	5.16	68.8
C4	1.08	0.46	42.6	2.16	2.90	134	15	9.71	64.7	15	10.5	69.7
C5	2.16	2.40	111	4.32	4.87	113	30	19.7	65.7	30	19.4	64.7
C6	4.32	4.0	92.6	8.64	8.18	94.7	60	41.2	68.7	60	46.8	78.1
C7	–	–	–	17.3	14.08	81.5	120	84.9	70.8	120	94.2	78.5
C8	–	–	–	–	–	–	240	169	70.5	240	176	73.2

of 42 kHz for 30 min. After this time, samples were centrifuged for 10 min at 4000*g* set at 20 °C. The supernatant was then collected with sterile syringes coupled to a Whatman 0.45 μm filter and packaged in vials for subsequent quantification. The chromatographic separation and mass spectrometry detection were performed with an UHPLC Nexera X2 Shimadzu coupled with a Triple TOF TM 5600+ from AB Sciex (UHPLC-ToF-MS). The UHPLC system consisted of a vacuum degasser, an autosampler with controlled temperature, a binary pump and an oven for the chromatographic column. The autosampler was kept at 10 °C and the injection volume was 10 μL. The ToF-MS ionization was performed with negative electrospray ion source (ESI-) mode with full-scan data acquisition from 100 to 920 Da and using the Analyst ® TF software. Identification and quantification were performed with the PeakView™ and MultiQuant™ software. The identification criteria followed was based on the exact mass with an error below 10 ppm, variation of relative retention time to a maximum of 2.5% and the isotope ratio difference lower than 10%. The TOF-MS detector was calibrated between each 10 injections to guarantee the accurate mass resolution. Limits of detection (LOD) and quantification (LOQ) for glyphosate were established at 0.1 and 0.5 mg kg^{-1}, respectively.

Chlorothalonil analytical determinations followed the methodology described by Singh et al. (2002), with some adaptations. Moist soil subsamples of 5 g were weighed into 15 mL sterile polypropylene centrifuge tubes, to which 4.5 mL of acetonitrile and 0.5 mL of ultrapure water were added. Samples were vortex mixed and placed in an orbital shaker for 1 h. Samples were then centrifuged at 5000*g*, for 5 min and the supernatant was filtered and injected to the HPLC system. HPLC-UV determination was conducted with a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155). The chromatographic column used for separation was an ACE C18 column (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA) equilibrated at 25 °C. Scanning a solution of chlorothalonil allowed the selection for the most accurate wavelength for elution monitoring. Maximum absorption was achieved at 325 nm. Chlorothalonil was analyzed in isocratic mode with a mobile phase constituted by 0.1% formic acid in water (70%) and acetonitrile (30%). Twenty μL of sample were injected, flowing at a rate of 1.2 mL/min for a total run time of 17 min. LOD and LOQ for chlorothalonil were 0.17 and 0.5 mg kg^{-1}, respectively.

2.6. Data analysis

For the reproduction tests, the LC50 values were calculated using probit analysis through IBM SPSS Statistics 24 software (IBM, New York, United States). Inhibitory effects on reproduction (EC50) were estimated using non-linear regression models (logistic, hormetic or exponential model depending on the best fit of data), using Statistica software (version 7, TIBCO, California, United States). Both LC50 and EC50 estimations presented in Table 2 used the actual concentrations measured through chemical analyses. For evaluating survival and reproduction effects, when normality and homoscedasticity assumptions were satisfied, significant differences between response means of treatments and controls were tested using one-way ANOVA analysis followed by post-hoc Dunnett's pairwise comparison tests. When ANOVA assumptions were not verified, the non-parametric Kruskal-Wallis test followed by post-hoc Dunn's test of multiple comparisons using ranked sums was used.

For the dissipation analysis (DT50), concentrations of pesticides (converted to percentage of the initial concentration) were plotted versus time. The FOCUS work group guidance document on degradation kinetics (FOCUS, 2006) was followed to assess the best kinetic models of dissipation for the different pesticides, using the least squares method with the SOLVER supplement from the Microsoft Office Excel® 2016 and *t*-tests. Two models were selected to fit dissipation kinetics, a simple first order (SFO) and a bi-phasic double first-order in parallel (DFOP), according to Eqs. (1) and (2):

$$\mathrm{SFO}: Ct = C0 \cdot e^{-kt} \tag{1}$$

$$\mathrm{DFOP}: Ct = C0 \cdot \left(g \cdot e^{-k1.t} + (1-g) \cdot e^{-k2 \cdot t}\right) \tag{2}$$

where Ct is the concentration (%) of pesticide remaining in soil at time t (days); C0 is the initial concentration (%) of pesticide; k is the rate of dissipation ($days^{-1}$); k1 and k2 are constant rates for the different compartments and g is the fraction of degradation occurring under rate constant k1. Normal distribution was evaluated with Kolmogorov-Smirnov tests in all treatments.

3. Results

3.1. Pesticides degradation

Residues of chlorothalonil or glyphosate were not found in control samples of the natural tested soil, thus validating its use in this study. The tested concentrations for both pesticides presented low persistence in the tested soil, with relatively fast degradation (low DT50 values) under aerobic conditions. The dissipation kinetics for pure and formulated glyphosate and chlorothalonil pesticides are presented in Fig. 1.

The initial recovery percentages ($t = 0$ days) for Montana® and glyphosate were 58.6 ± 2.7 and 57.6 ± 2.4. For Bravo 500® and chlorothalonil, these percentages were 67.6 ± 1.7 and 72.7 ± 1.6, respectively. The herbicide glyphosate presented half-life values of 2.2 days (95% CI: 1.1–4.9 days) in the formulation and 2.8 days (95% CI: 1.9–5 days) as pure active substance. Chlorothalonil presented half-life values of 1.1 days (95% CI: 0.78–1.3 days) in the tested formulation and 2.9 days (95% CI: 2.6–3.3 days) as pure compound (Fig. 1). The estimated degradation rates were faster when both chemicals were evaluated in their respective formulations than in pure forms. For the active ingredient chlorothalonil, degradation was not complete after 30 days (about 20% of initial concentration remaining), while for the formulation there was a complete degradation observed after 7 days of application in the tested soil. The dissipation kinetics followed distinct patterns for the fungicide, a DFOP (Double first order in parallel, $r^2 = 0.98$, $\chi^2 = 3.9$, $p = 0.14$) for the pure chlorothalonil and a SFO (Single first order, $r^2 = 0.95$, $\chi^2 = 4.0$, $p = 0.14$) for the formulation. As for the herbicide, a SFO dissipation pattern was fitted better for both treatments ($r^2 = 0.81$, $\chi^2 = 14.8$, $p = 0.002$ for Montana® and $r^2 = 0.65$, $\chi^2 = 20.1$, $p < 0.001$ for glyphosate). All datasets followed a normal distribution ($p > 0.05$).

3.2. Effects on reproduction

All reproduction tests fulfilled the validity criteria described by the standard guideline (ISO, 1999), with adults survival higher than 80%, number of juveniles higher than 100 per replicate, and coefficient of variation of reproduction lower than 30% in control replicates. Acetone treatment presented no influence, neither on survival ($p = 0.101$) nor reproduction ($p = 0.121$). Therefore, EC50 and LC50 values were estimated with the non-treated control samples. The toxic values estimated in each reproduction tests are presented in Fig. 2.

For Montana® formulation, the estimated EC50 of the formulation on reproduction was 4.63 mg (a.i.) kg^{-1} (95% CI: 0.58–8.68 mg kg^{-1}), by fitting to a logistic model ($R^2 = 0.52$). The formulation presented no effects on survival with the tested range of concentrations in this experimental setup (Fig. 2). As for the pure active substance glyphosate, no effects on *F. candida* survival or reproduction were observed, even for the additional concentrations ($F_{7,32} = 0.85$, $p = 0.55$ and $F_{7,32} = 1.17$, $p = 0.35$, respectively).

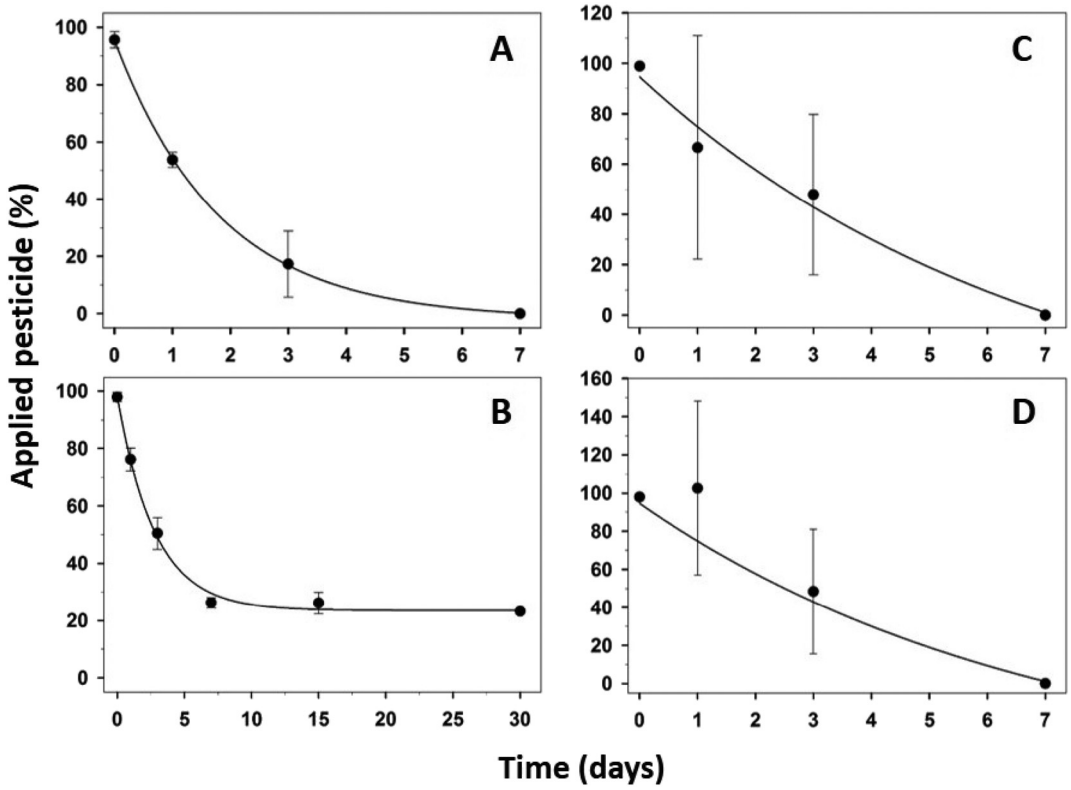

Fig. 1. Degradation kinetics for glyphosate and chlorothalonil (formulated and as pure active substances) in a natural agricultural soil, under aerobic conditions. Pesticides were spiked in their formulated and pure forms, at final concentrations of 5 mg (a.i.) kg^{-1}. A - Bravo500®; B - Chlorothalonil; C - Montana®; D - Glyphosate. Results are expressed in percentage relatively to initial concentrations.

In an attempt to find effect concentrations for the active ingredient glyphosate, several dilutions [up to 900 mg (a.i.) kg^{-1}] were tested in a new reproduction test (data not shown) with, however, no significant effects registered for reproduction or survival in any of the tested concentrations.

For the fungicide formulation, Bravo500®, the effect on reproduction fitted better a hormesis model ($r^2 = 0.92$), representing a biphasic effect. At a first stage, there seems to be stimulation in reproduction with the lower concentrations of the fungicide, with consequent inhibition with higher doses at a second phase. The estimated EC50 was 41.3 mg (a.i.) kg^{-1} (95% CI: 30.9–51.7 mg kg^{-1}). The effect of the pure active substance chlorothalonil on *F. candida* reproduction presented a similar trend to the formulation, also fitting a hormesis dose-response model ($r^2 = 0.96$), revealing however stronger effects than the formulation (lower EC50), being the estimated EC50 14.9 mg (a.i.) kg^{-1} (95% CI: 12.5–17.4 mg kg^{-1}). Effects on survival of *F. candida* were also observed for both forms of the fungicide (formulation and pure form). The estimated LC50 values for the formulation and the active ingredient were 117 mg (a.i.) kg^{-1} (95% CI: 97.1–147 mg kg^{-1}) and 73.5 mg (a.i.) kg^{-1} (95% CI: 59.2–93.9 mg kg^{-1}), respectively. The pure active ingredient was more toxic to the reproduction of *F. candida* than the formulation.

4. Discussion

4.1. Pesticides degradation

Glyphosate is considered to be semi-persistent in soil, but its persistence may differ widely, with reported half-life (DT50) ranging between 1 and 197 days (Bento et al., 2016), normally following first order degradation kinetics, as verified in the present study. Testing a glyphosate concentration of 16 mg kg^{-1}, which was three times higher than the concentration used in the present study, Bento and co-authors (2016) reported half-lives (DT50) between 1.5 and 10.8 days when analyzing degradation of glyphosate at higher temperatures (30 °C) in a natural non-sterile soil. Using a WHC of 60% (similar to our study), the authors reported DT50 values of 1.8 and 2.2 days. This is also in agreement with the results obtained here. Its degradation in soils is influenced by many spatially variable factors like soil properties, which highlights the importance of performing ecotoxicological tests using natural soils, as addressed in this study. Its dissipation in soil has been reported as occurring mainly by microbial degradation (Bento et al., 2016; Kim et al., 2011; La Cecilia and Maggi, 2018). Several soil microorganisms have demonstrated capabilities to degrade glyphosate, using it as a source of phosphorus (Sviridov et al., 2015). Abiotic factors such as temperature, pH, or organic matter may also contribute, but in a lesser extent (Barrett and McBride, 2005). Since the degradation tests of the present study were performed under aerobic conditions and using a natural soil, a considerable microbial activity was expected to occur over the test, fact that was supported by the high degradation rates observed.

The influence of certain chemical compounds in soil in glyphosate persistence is still not completely known and data from the literature has been contradictory in some cases. For instance, despite glyphosate dissipation in soil has been reported as negatively correlated to metals such as copper (Barrett and McBride, 2005; Kim et al., 2011), a recent study using 21 agricultural soils revealed no such correlations (Nguyen et al., 2018). On the other hand, the authors demonstrated that glyphosate mineralization revealed a positive correlation with calcium (Ca^{2+}). Therefore, given the relatively high richness of the natural soil in calcium (5400 mg kg^{-1}; Table 1) most probably it also contributed to the high degradation rates observed.

Chlorothalonil has been considered a very low to moderately persistent fungicide in soil, with reported DT50 in the range from a few days to 2 months (EFSA, 2018), normally following single first order and biphasic kinetics of dissipation. Both situations were confirmed in the present study. Contrarily to glyphosate and other pesticides, the wide

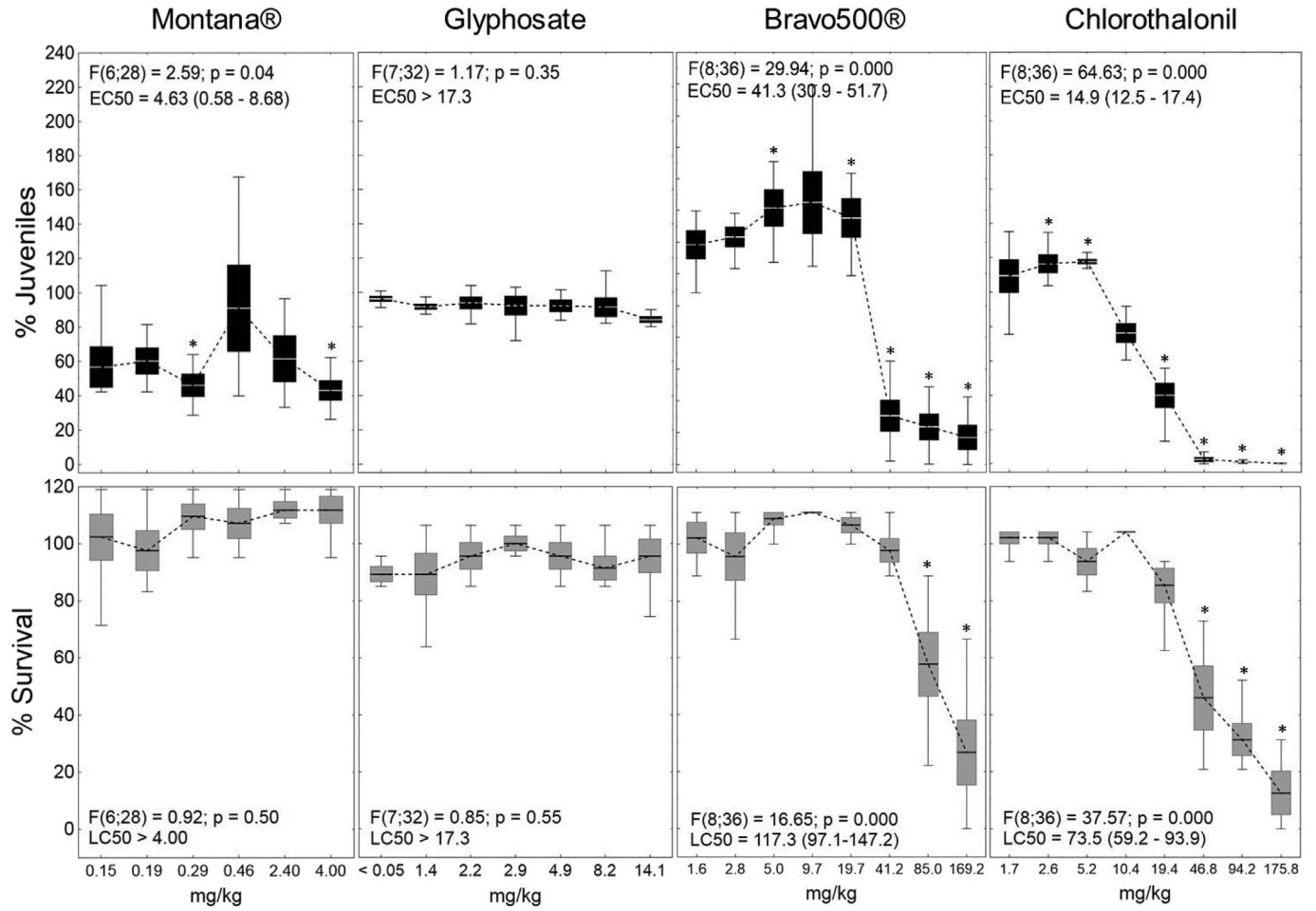

Fig. 2. Effects on reproduction (first line of graphs) and survival (second line of graphs) of Montana® and Bravo500® formulations and their respective pure active ingredients (glyphosate and chlorothalonil) on *Folsomia candida* exposed in a natural agricultural soil. Estimated EC50 and LC50 values are shown (expressed in mg·kg^{-1} of dry soil). Results are presented in percentage (%) of control. * Significant differences compared with the control ($p < 0.05$).

degradation ranges reported for chlorothalonil may depend on abiotic factors. The influence of pH and organic matter were demonstrated to play a determinant role in this process (Bending et al., 2007; Wang et al., 2011; Wu et al., 2012), followed by the influence of microbial activity that although in less extent still has a relevant influence (De Souza et al., 2017).

The half-life (DT50) values found for chlorothalonil are in accordance with results reported in the literature (De Souza et al., 2017; Chaves et al., 2007). Also using a natural soil, De Souza et al. (2017) reported dissipation rates of <1 day for chlorothalonil, using a concentration of 1.385 mg (a.i.) kg^{-1} soil, in the same order of magnitude as the one evaluated in the present study. Using similar concentration ranges, Chaves et al. (2007) pointed out that 44% of the applied chlorothalonil (in the formulation Bravo 720®) was dissipated in the first hours after its application in a tropical soil, reporting a final half-life of 2.2 days. The latter authors observed strong interactions between chlorothalonil and the organic fraction of the soil, forming non-extractable residues and thus constituting an important route of dissipation, as previously discussed (Gamble et al., 2000; Wang et al., 2011). In agreement with the data presented here, such results reflect the high sorption potential of chlorothalonil and its reported low mobility. Degradation values (DT50) were in general similar for both pesticides. However, while chlorothalonil degraded completely after 7 days (DT50 = 1.1 days) when applied as formulation, its pure form degraded only 80% after 30 days (DT50 = 2.9 days), presenting different degradation kinetics and rates. Formulated pesticides can have a different degradation rate from pure compounds (Benoit et al., 2015; Huston and Pignatello, 1999). For pure chlorothalonil, the relatively fast and stable sorption rates to the soil matrix could lead to a consecutive decrease in degradation, comparatively to the formulated product. Possibly, the added co-formulants in the mixture reduced the sorption rates, or even the chemical stability of the chemical, leading to a faster decay. This was previously demonstrated for other chemicals (Kah et al., 2016). Such results may be linked to the distinct effects observed on survival and reproduction of *F. candida* when the formulation or the isolated active ingredient was applied, as discussed below.

4.2. Effects on reproduction

According to Von Mérey et al. (2016), the maximum glyphosate concentrations generally predicted in soil, assuming application to permanent crops (tillage depth of 5 cm) at the maximum cumulative annual application rate of 4.32 kg glyphosate (a.i.) ha^{-1} is reported to be 6.62 mg kg^{-1} dry soil. The reproduction EC50 found here for *F. candida* [4.63 mg (a.i.) kg^{-1} of soil; Fig. 2] has thus high ecological relevance, since effects on population of these key arthropods can be expected with exposures to the recommended application dose of similar formulations. The EC50 range estimated here was almost 15× higher than that reported by Santos et al. (2010), where the authors found a reproduction EC50 of 0.33 mg (a.i.) kg^{-1} after exposing *F. candida* to the same glyphosate formulation, although in a standardized natural soil (LUFA 2.2). In a more recent study (Niemeyer et al., 2018), the effects in reproductive fitness of *F. candida* were tested for four commercial glyphosate formulations, also using a natural soil collected in the field.

The authors did not find significant changes on reproduction for any of the tested formulations until the concentration of 69.8 mg (a.i.) kg^{-1}. The discrepancy of results reported in different studies is most probably related to the influence of different soil types on activities of contaminants. This highlights the importance of measuring soil properties, which may affect pesticide activities. Although studies addressing glyphosate effects on other soil invertebrates are limited, *F. candida* seems to be more sensitive to glyphosate formulations than other invertebrates. This model organism demonstrated to be more sensitive to glyphosate formulations than, for instance, the earthworm *Eisenia Andrei* and the isopod *Porcellio dilatatus*, after following single and multispecies avoidance tests (Niemeyer et al., 2018; Santos et al., 2012).

For the pure active substance glyphosate, no effects on survival or reproduction were found for *F. candida* (Fig. 2) even after exposure to very high concentrations up to 900 mg kg^{-1} (results not shown). One possible explanation for the absence of significant effects could be the chelating properties of the chemical (Duke et al., 2012). By complexing with metals naturally present in the soil, it can possibly become inactive and therefore decrease its toxicity to the organisms. This was also reported and discussed by Von Mérey et al. (2016), exposing earthworms, springtails, and predatory mites to pure glyphosate, although in a standard OECD soil. The authors reported a NOEC of 472.8 mg kg^{-1} dry soil (the highest concentration tested) and therefore no effects on reproduction of collembolans.

The effects of the herbicide formulation, however, presented significant effects on reproduction of *F. candida* and, considering the results with the pure substance, it is therefore unlikely that the observed effects were due to glyphosate. Moreover, degradation rates observed for pure and formulated glyphosate were similar (2.2 and 2.8 days), which makes it difficult to attribute the effects to the pure active ingredient, since these were observed only when the formulation was applied. In a study conducted by Casabé et al. (2007), the related glyphosate-based formulation, ROUNDUP® FG, applied in a natural soya soil, was demonstrated to cause a decreasing number of juveniles, but also avoidance behavior and reduced cocoon viability in the earthworm *Eisenia fetida*. More recently, also reproduction success of other two earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*) was demonstrated to decrease significantly within three months after sequential application of two Roundup® formulations, below the recommended dosage (Gaupp-Berghausen et al., 2015). Similarly to these formulations, the one tested in the present study is composed by other chemicals, namely a polyethoxylated tallow amine adjuvant (POEA), as mentioned in Section 2.1. However, the exact POEA concentration present in the formulation is not mentioned in the commercial package (range from 7 to 12% p/p). Ethoxylated adjuvants are lipophilic substances with alkyl chains and/or ethoxy-groups in the ethoxylated chains, which can penetrate cell membranes disrupting their structure and functions (Nobels et al., 2011). Despite the scarce information about the effects of this adjuvant in terrestrial invertebrates, POEA has been reported, in the last few years, to present even higher toxicity than glyphosate alone, on different models such as rats, amphibians, crustaceans, fish, bacteria, and human cells (Mesnage et al., 2015). In 2015, the European Food Safety Authority released a report with similar conclusions (EFSA, 2015). The formulation used in this study was acquired prior to the decision from the EC to withdraw POEA from glyphosate-based products in 2016 (MEMO-16-2012). However, the POEA containing herbicide formulations are still used outside Europe (Tush and Meyer, 2016). There is still high potential for their release into the environment and it urges to understand the real environmental adverse effects and toxicity mechanisms of such chemicals on non-target soil organisms.

According to the predicted data generated from EPI (Estimation Programs Interface) Suite™ predictive model (available at www.chemspider.com), glyphosate revealed high solubility in water [S_w (25 °C) = 1×10^6 mg L^{-1}] and low soil sorption coefficient (Koc_{log} = 1.27). Comparatively to the herbicide, chlorothalonil presents low solubility [S_w (25 °C) = 26.01 mg L^{-1}] and, therefore, increased affinity for the soil organic matter (Koc_{log} = 3.38). As a consequence, the route of exposure for *F. candida* via contaminated-food ingestion must also be considered here. This could explain the observed higher adverse effects on survival and reproduction of *F. candida*, comparatively to glyphosate. The estimated EC50 effects on reproduction of *F. candida* upon exposure to the fungicide formulation [41.3 mg (a.i) kg^{-1}], were in accordance with the results reported by Leitão et al. (2014), where the authors (using a similar natural soil) reported a reproduction EC50 value of 31.1 mg (a.i.) kg^{-1}. In the same study, *F. candida* also demonstrated to be the most sensitive species to the fungicide, among three other invertebrate species, thus confirming *F. candida* as a sensitive and suitable species for testing toxicity of such chemicals. Despite studies on the ecotoxicological effects of chlorothalonil in soil invertebrates are scarce, there are some reports on its effects in reproduction of aquatic organisms. Chlorothalonil exposure caused delayed hatch in sockeye salmon (*Oncorhynchus nerka*), by exposing the animals to a concentration of 5 μg/L (a.i.), using the same formulation as the one used in the present study (Du Gas et al., 2017). In *Daphnia magna,* the concentration of 0.72 mg L^{-1} (a.i.) of this formulated fungicide prolonged the first pregnancy and first brood times (Song et al., 2017). In fact, this chemical has been reported to be extremely toxic to organisms, exhibiting even carcinogenic effects upon exposure (Elskus, 2012). The exposure to pure chlorothalonil in the present study revealed stronger effects in reproduction and survival of collembolans than the formulation. The higher toxicity observed for the active ingredient is possibly due to a slower degradation of chlorothalonil. Thus, results suggest that the effects observed for the formulation were caused by chlorothalonil (a.i.).

As previously considered by Newman et al. (2006), the present work highlights the ecological importance of considering the formulated products when evaluating fate and effects of pesticides, since these may present different characteristics from those of the isolated chemicals. Although the use of natural agricultural soils bring relevant data to be considered and compared with available work, further comparative studies between natural and artificial soils need to be addressed in the literature for these and other chemicals.

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