Applied Soil Ecology 76 (2014) 124–131

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil

Sara Leitão[a,*], M. José Cerejeira[a], Paul J. Van den Brink[b,c], José Paulo Sousa[d]

[a] Instituto Superior de Agronomia, University of Lisbon, Centro de Engenharia dos Biossistemas (CEER), Tapada da Ajuda, 1349-017 Lisboa, Portugal
[b] Department of Aquatic Ecology and Water Quality Management, Wageningen University, Wageningen University and Research centre, P.O. Box 47, 6700AA Wageningen, The Netherlands
[c] Alterra, Wageningen University and Research centre, P. O. box 47, 6700AA Wageningen, The Netherlands
[d] IMAR-CMA, Department of Life Science, University of Coimbra, Apartado 3046, 3001-401 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form
20 December 2013
Accepted 27 December 2013

Keywords:
Pesticides
Non-target soil organisms
Natural soil
Mediterranean conditions
Ecotoxicity

ABSTRACT

The potential terrestrial toxicity of three pesticides, azoxystrobin, chlorothalonil, and ethoprophos was evaluated using reproduction ecotoxicological tests with different non-target species: the collembolan *Folsomia candida*, the earthworm *Eisenia andrei*, and the enchytraeid *Enchytraeus crypticus*. All reproduction tests were performed with natural soil from a Mediterranean agricultural area (with no pesticide residues) in order to improve the relevance of laboratory data to field conditions. Controls were performed with natural and standard artificial soil (OECD 10% OM). The fungicide azoxystrobin showed the highest toxicity to earthworms (EC_{50} = 42.0 mg a.i. kg^{-1} dw soil). Collembolans were the most sensitive taxa in terms of sublethal effects of chlorothalonil with an EC_{50} of 31.1 mg a.i. kg^{-1} dw soil followed by the earthworms with an EC_{50} of 40.9 mg a.i. kg^{-1} dw soil. The insecticide ethoprophos was the most toxic to collembolans affecting their reproduction with an EC_{50} of 0.027 mg a.i. kg^{-1} dw soil. Enchytraeids were generally the least sensitive of the three species tested for long-term effects. Earthworms were not always the most sensitive species, emphasizing the need to increase the number of mandatory assays with key non-target organisms in the environmental risk assessment of pesticides.

1. Introduction

The environmental risk assessment (ERA) of pesticides is based mainly on scenarios developed for northern and central European conditions. This may pose a problem when used for Mediterranean conditions where soil properties, climatic conditions, biological communities, agricultural practices, and crops are substantially different (Daam et al., 2011a; Ramos et al., 2000). These generic scenarios can over- or underestimate the real risks of pesticides when applied to a typical Mediterranean environment (Ramos et al., 2000). Therefore, the use of natural soils is becoming more and more important when performing relevant regional ERA among European regions (Chelinho et al., 2011). Pesticides ERA for terrestrial organisms uses standardized ecotoxicological tests traditionally performed in standard artificial soil (e.g., OECD, 1984; ISO, 1998), or in standard natural soil (e.g., LUFA2.2) that often do not possess the characteristics of agricultural natural soils, therefore not mimicking realistic exposure conditions to pesticides for soil biota in the field (Kuperman et al., 2006). It has been documented that differences in soil properties, such as for e.g., organic matter content, may influence pesticide persistence in soil and bioavailability to soil-dwelling organisms (enchytraeids and earthworms) (Amorim et al., 2002a,b; De Silva et al., 2009; Kuperman et al., 2006). Compared to standard artificial soils, natural soils may have properties supporting higher bioavailability of test chemicals; as such their use considerably improves the relevance of laboratory ecotoxicological data for field conditions (Kuperman et al., 2006; Van Gestel et al., 2011). Therefore, the importance of using natural soil is supported by the need to develop more realistic ecotoxicological evaluations for terrestrial ecosystems.

Until the implementation of the new data requirements setup according to the new Pesticide Regulation 1107/2009 (EU, 2013), the protection of terrestrial ecosystems at a first-tier level is assessed in the ERA of pesticides using only the earthworms acute

* *Corresponding author at*: Instituto Superior de Agronomia, University of Lisbon, Centro de Eng, Departamento de Ciências e Engenharia de Biossistemas (DCEB), Tapada da Ajuda, 1349-017 Lisboa, Portugal. Tel.: +351213653222.
E-mail addresses: saraleitao@isa.utl.pt, saraleitao@yahoo.com (S. Leitão), mcerejeira@isa.utl.pt (M.J. Cerejeira), Paul.vandenBrink@wur.nl (P.J. Van den Brink), jps@zoo.uc.pt (J.P. Sousa).

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http://dx.doi.org/10.1016/j.apsoil.2013.12.013

test with *Eisenia fetida sensu lato* (*E. fetida* and *E. andrei*) (EC, 2009; SANCO, 2002). Tests using other non-target organisms can be performed if non-target arthropods are believed to be at risk, e.g., tests with Collembola and mites, and on a case-by-case basis depending on the type of the pesticide and its application method (SANCO, 2002). Although earthworms are key species of terrestrial ecosystems as decomposers contributing significantly to organic matter decomposition, nutrient cycling and soil formation (Edwards and Bohlen, 1992; EFSA, 2009a), there is a need for further tests evaluating sub-lethal effects on soil organisms from different trophic levels, taxonomic, physiological and/or functional groups in order to improve the ERA of chemicals in soil (Daam et al., 2011b; EFSA, 2010a; Frampton et al., 2006; Römbke and Moser, 2002). Although there is a growing concern about the potential adverse effects of pesticides in the environment, there is a lack of sub-lethal ecotoxicity data available for non-target terrestrial invertebrates (Daam et al., 2011b; Frampton et al., 2006).

Thus to overcome these limitations, this study aimed to: (i) evaluate sub-lethal effects of pesticides with different types of toxic action (two fungicides and one insecticide) on the reproductive performance of non-target soil invertebrates from different trophic levels: collembolan, enchytraeids, and earthworms; (ii) to increase knowledge on pesticides behavior in the environment (e.g., fate, bioavailability), by using a natural soil from a Mediterranean agricultural area, and (iii) to perform a first-tier risk characterization for the three pesticides by comparing the obtained toxicity data with reported exposure data, whenever possible, elucidating the importance of using natural soil when evaluating exposure and effects on terrestrial organisms.

2. Material and methods

2.1. Pesticide selection, characterization, spiking, and analytical procedures

Two fungicides, azoxystrobin and chlorothalonil, and the insecticide ethoprophos were chosen after a selection from a list of pesticides authorized on irrigated crops (onion, maize, and potato) in Portugal. A preference was given to insecticides and fungicides with high expected toxicity to soil organisms (Frampton et al., 2006; Wang et al., 2012). The selection was based mainly on ecotoxicity data to terrestrial organisms, namely to earthworms, due to the lack of information on collembolans and enchytraeids. Relevant intrinsic physical and chemical characteristics such as water solubility, capacity to adsorb to soil particles, volatilization, and persistence in soil were also taken into account (Table 1). Information on environmental fate, such as the potential for leaching into groundwater and the predicted environmental distribution (PED) (Table 1), focusing on the soil and water compartments, was assessed using the Groundwater Ubiquity Score and the Mackay fugacity model, respectively (Gustafson, 1989; Mackay, 2001). The application mode (e.g. direct soil application) was also taken in account.

Azoxystrobin (CAS 131860-33-8; methyl (*E*)-2-{2-[6- (2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate) is a strobilurin fungicide with protectant, curative, eradicant, translaminar, and systemic properties. Its mode of action focuses on inhibiting mitochondrial respiration, spore germination, and mycelial growth and also showing antisporulant activity. It possesses a broad spectrum of activity against the four major groups of fungi: Ascomycota, Oomycota, Deuteromycota, and Basidiomycota (Bartlett et al., 2002; MacBean, 2012). It has been identified as low toxic to birds, mammals, bees, and other non-target terrestrial organisms (arthropods and earthworms) (Bartlett et al., 2002; Gullino et al., 2000).

Table 1
Pesticides physico-chemical characteristics, environmental potential fate (Groundwater Ubiquity Score—GUS and Predicted Environmental Distribution—PED), and pesticides ecotoxicity data for terrestrial earthworm (all data from MacBean, 2012 unless indicated otherwise).

	Azoxystrobin	Chlorothalonil	Ethoprophos
S_w (mg L^{-1})	6.0	0.81 (25 °C)	700
K_{ow} (Log P)	2.5 (20 °C)	2.9 (25 °C)	3.59 (21 °C)
K_{oc} (ml g^{-1})	690[a]	850[d]	111[f]
VP (mPa)	1.10E-07	0.076 (25 °C)	78[f]
DT_{50}lab soil (d)	279[b]	0.3–87[d]	10–25[f]
DT_{50} field soil (d)	14	18–70[d]	4–25[g]
GUS	2.84	2.08 (DT_{50}lab soil 87)	2.73 (DT_{50}lab soil 25)
PED (%)			
Soil	49.5	43	76.1
Air	7.39E-08	0.285	0.122
Aerossol	8.72E-03	2.08E-03	5.91E-05
Water	49.3	55.7	22.1
Sediment	1.10	0.955	1.69
Suspended solids	0.0344	0.0299	0.0528
Aquatic biota	1.23E-05	2.43E-03	4.29E-03
Earthworms (lethal tests) LC_{50} (14d) (mg kg^{-1})	283	>404/268.5 (5% OM)[d]	39.6[f]
NOEC (14 d) (mg kg^{-1})	20[c]	25 (5% OM)[d] 1,65 (5% OM)[d,e]	<1.67 (56 d)[f]

S_w—Solubility in water at 20 °C; K_{ow}—Octanol-water partition coefficient at pH7; K_{oc}—Organic carbon sorption constant; VP—Vapor pressure at 20 °C; DT50—Half life in soil at 20 °C under aerobic conditions; GUS = log(DT50) × (4—log(K_{oc})) – GUS > 2.8: leacher; 1.8 < GUS < 2.8: transition; GUS < 1.8: improbable leacher (Gustafson, 1989); PED—Predicted Environmental Distribution according to Mackay (2001)—Mackay fugacity model ('level I, version 3.00, 2004, Trentu University, Canada') PED < 20%: very low affinity; 20% ≤ PED < 40%: low affinity; 40% ≤ PED < 60%: average affinity; 60% ≤ PED < 80%: high affinity; PED ≥ 80%: very high affinity; OM—organic matter.

[a] EFSA (2010b), value for sandy clay loam soil.
[b] EC (1998), average value resulting from different soils.
[c] FOOTPRINT (2012).
[d] EC (2006).
[e] EC (2006), test with chlorothalonil 500 g L^{-1} SC.
[f] EFSA (2006).
[g] EFSA (2006), representative range for southern and central Europe locations.

Chlorothalonil (CAS 1897-45-6; tetrachloroisophthalonitrile) is a chloronitrile fungicide with a non-systemic broad-spectrum mode of action and foliar action with some protectant properties. It is a broad spectrum organochlorine fungicide effective against fungal diseases like potato late blight agent and fungus *Phytophthora infestans* (Mont.) de Bary and *Alternaria solani* (Ellis & G. Martin) L.R. Jones & Grout. Chlorothalonil acts also by preventing spore germination and zoospore motility (Sakkas et al., 2002; MacBean, 2012). Although effects on earthworms have been registered (Potter et al., 1994; Tu et al., 2011), information on other non-target organisms is scarce.

Ethoprophos (CAS 13194-48-4; *O*-ethyl *S*,*S*-dipropyl phosphorodithioate) is a broad spectrum organophosphate insecticide and nematicide with moderate residual activity and is not phytotoxic. It is an acetylcholinesterase inhibitor and is a non-systemic nematicide and soil insecticide with contact action. Ethoprophos is effective against potato nematodes (*Globodera rostochiensis* (Wollenweber) Behrens, *G. pallide* (Stone) Behrens) and soil insects (*Agriotes* spp., *Agrotis* spp. and *Melolontha* spp.) on maize crop (Karpouzas et al., 1999a,b; MacBean, 2012). Effects on non-target soil organisms are scarce and the information available is related to artificial soil (EFSA, 2006; Sánchez-Moreno et al., 2009), although effects on terrestrial arthropods may be expected due to the pesticide type of toxic action (Frampton et al., 2006). Adverse effects on the abundance and biomass of earthworms are known (reduction of 88–95% and 83–96%, respectively, 3 weeks after the application of 5.6 kg a.i. ha^{-1} of Mocap10G in turf soil) (Potter et al., 1994).

In order to evaluate the environmental impact of the pesticides under realistic application in the agricultural fields, azoxystrobin, and chlorothalonil were tested as the concentrated suspension formulation ORTIVA® (250 g a.i L^{-1}) and BRAVO 500® (500 g a.i. L^{-1}), respectively. Ethoprophos was tested as pure compound (Dr. Ehrenstorfer 93.0% purity) because the available formulation in Portugal (MOCAP 10G®) consists of microgranules which poses a limitation in terms of nominal concentration calculation since it remains active in soil against insects for 2–4 months. For spiking procedures, specific amounts of the aqueous solution of each pesticide were prepared with distilled water to attain a moisture content of the natural soil of 50% of the water-holding capacity (WHC). No solvents were used due to the fungicides formulations, and the insecticide active ingredient, high solubility in water. The soils were spiked on day one of the start of the experiments and the aqueous solutions used for spiking the soil were stored in refrigerated conditions (4–6 °C) until pesticide residue analysis. Azoxystrobin and chlorothalonil residues in water were analyzed by an independent laboratory, through solid phase extraction followed by gas chromatography/mass spectrometry (SPE/GC–MS) and ethoprophos residues through by liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) according to DIN 38407-F 2 (1993) and ISO 10695 (2000). Limits of quantification (LOQ) were 0.1 μg ml^{-1} for azoxystrobin, 0.3 μg ml^{-1} for chlorothalonil and 0.05 μg L^{-1} for ethoprophos.

2.2. Test organisms and culture conditions

Three different soil organisms were used: springtails *Folsomia candida* (Willem, 1902) (Collembola: Isotomidae), the potworm *Enchytraeus crypticus* (Westheide & Graefe, 1992) (Oligochaeta: Enchytraeidae) and the earthworms *Eisenia andrei* (Bouché, 1972) (Oligochaeta: Lumbricidae). All test organisms used in the experiments originated from laboratory cultures maintained at a constant temperature of 20 ± 2 °C with a photoperiod of 16:8 h light:dark. Springtails were cultured in plastic vessels lined with an 11:1 mixture of plaster and activated charcoal. A small amount of granulated dry yeast was added as a food source once a week to avoid spoilage by fungi and moldy food was removed when detected. The organisms were synchronized to be 10–12 days old at the start of the test. The Enchytraeid *E. crypticus* is listed in the ISO protocol 16387 (2004) as an alternative to *E. albidus* and was chosen for this study due to its better performance on natural soils with pH, organic matter content (OM), and clay characteristics similar to the test soil. It is also the preferred species when assessment objectives include natural soil types that support higher bioavailability of chemicals (Kuperman et al., 2006). The enchytraeids were cultured in aerated plastic vessels using uncontaminated garden soil that was defaunated before use by deep-freezing cycles and with no additives as compost of fertilizers and pesticides. The soil was moistened at 50% WHC and verified weekly to maintain its moisture content. The test organisms were fed weekly with finely ground dry oat placed under soil particles to prevent fungal growth and facilitate availability of food to small juveniles (Römbke and Moser, 2002). The organisms used in the tests were carefully removed from the soil with the help of tweezers and placed on petri dishes with distilled water for selection under a stereomicroscope, as possessing clitella and a body size between 10 mm and 12 mm long. Before the performance of the experiments the natural test soil was checked for its suitability by observing the response behavior of a group of organisms for a period of more than 2 weeks (Römbke and Moser, 2002). Earthworms were kept in aerated plastic vessels with a mixture of horse manure and peat as substrate. This mixture was moistened periodically to maintain the moisture content between 40% and 60% of the WHC. The test organisms were fed twice a month with oat porridge. The earthworms used in the tests were synchronized to be more than one month old and before the start of the experiments the adults with clitella were separated and acclimated to the uncontaminated test substrate (natural soil and OECD 10% OM) for a period of between 24 h and 48 h. No mortality was observed during acclimation. After that, each organism was cleaned in water to remove soil particles, gently dried on absorbent paper, weighted (250–600 mg) and placed into plastic vessels covered with a lid in groups of ten.

2.3. Test soils

Artificial OECD soil with 10% organic matter content was prepared following the guideline instructions (OECD, 1984) and soil pH was adjusted to 6.0 ± 0.5 with $CaCO_3$. The natural soil used in this study, a eutric cambisol (EuDASM, 2011), is from an uncontaminated non-cultivated local from an important agricultural area in Ribatejo, Central Portugal. The uppermost soil layer (top 15–20 cm) was taken from the field, air dried, and sieved through a 2 mm mesh and submitted to several deep-freezing (−20 °C) cycles to eliminate any existing fauna, and preserved at 4–6 °C until used in the ecotoxicological tests. The soil was also tested for pesticide residues using a multi method ASU L 00.00–34 GC detection analyses (ASU L, 1999). Soil parameters measured in the laboratory were soil pH (1 M KCl), moisture content and water-holding capacity. Organic matter content, soil particle size distribution, cation exchange capacity, total elements, and other chemical and physical characteristics were assessed by international and internal laboratorial standard methodologies. The characteristics of the natural soil and methodologies used are summarized in Table 2. Both soils were moistened to 50% of the water-holding capacity immediately before the start of the tests.

2.4. Experimental design of terrestrial ecotoxicity tests

All test treatments were performed with natural soil and two control soil types were used, one with natural soil for results comparison and other with OECD artificial soil for organism's performance validation. The ecotoxicological tests were performed under a controlled temperature of 20 °C ± 2 °C with a light:dark cycle of 16 h:8 h.

2.4.1. Collembolan reproduction test

Chronic toxicity tests followed ISO (1999) procedures. The following gradients of concentrations were selected to assess the full dose-response relationships for each pesticide: azoxystrobin (10, 15, 20, 35, 50, 80, 120, 200, 300, 450, 650, and 1000 mg a.i. kg^{-1} dw soil); chlorothalonil (0.5, 1, 1.5, 2.5, 5, 10, 20, 30, 50, 80, 150, and 200 mg a.i. kg^{-1} dw soil) and ethoprophos (0.015, 0.020, 0.030, 0.040, and 0.050 mg a.i. kg^{-1} dw soil). The 28 days reproduction toxicity tests consisted of 10 synchronized springtails of 10–12 days old exposed to 30 g fresh weight soil per glass vessel, and fed with 2 mg of dry yeast at the start of the experiment. To reduce evaporation and prevent springtails from escaping, the vessels were closed with a lid with small holes to allow aeration. Two replicates were used per test concentration and not the standard four replicates due to the high number of concentrations adopted in order to increase the robustness of the test and EC_{50} calculations. Four replicates were used for each of the control soils as well as for the analysis of ethoprophos due to the number of concentrations tested. An extra vessel without test organisms and food was prepared for each combination and used for pH (1 M KCl) and moisture determination at the end of the test (ISO, 1994, ISO, 1999). All replicates were aerated twice a week, and 14 days after the start of the test 2 mg of granulated dry yeast were added and moisture loss replenished if needed, according to total initial vessel weights. After 4 weeks, juveniles were assessed by flooding the vessels with water, by adding a few

Table 2
Natural soil properties and respective analytical methods.

	Natural soil	Methods		Natural soil	Methods
Particle size distribution		Hydrometer of Boyoucos, IM	OM content (%)	5.74	Dry combustion ISO 10694, 1995
Sand (%)	54.4	–	Chemical parameters		–
Silt (%)	22.1	–	P_2O_5 (mg kg^{-1})	99	Egner–Rhiem ICP-OES, IM
Clay (%)	23.5	–	K_2O (mg kg^{-1})	>200	–
Soil texture	Sandy clay loam		Mg(mg kg^{-1})	>125	Ammonium acetate 1 M pH 7 FAAS, IM
pH (H_2O)	5.9	Potentiometry (20 ± 2 °C) IM, LAS.PL.20.V01, 2009	$CaCO_3$ (%)	0	ISO 10693, 1995
pH (1 M KCl)	5.0	ISO 10390 (1994)	Fe (mg kg^{-1})	>80	AAAc - EDTA (Lakanen)/FAAS, IM
Moisture (%)	11	ISO 11268–2.2 (1998)	Mn (mg kg^{-1})	38	–
WHC max (% dry weight)	54.4	ISO 11268–2.2 (1998)	Zn (mg kg^{-1})	1.8	–
Cation exchange capacity (me/100 g)	9.12	ammonium acetate 1 M pH 7 FAAS	Cu (mg kg^{-1})	3	–
Sum of base exchange (me/100 g)	6.72	(Ca and Mg) and FAES (K and Na) Titration IM	B (mg kg^{-1})	0.69	Boiling water/ICP-OES, IM
Degree of base saturation (%)	73.7	–	N (%)	0.297	Dry combustion ISO 13878, 1998

IM—internal method; WHC—water-holding capacity; FAAS—Flame atomic absorption spectrometry; FAES—Flame atomic emission spectrometry; OM—Organic matter; ICP-OES—Inductively coupled plasma optical emission spectrometry.

drops of ink and gentle stirring, after which the animals floating on the water surface were photographed and counted using the Image Tool software (Wilcox et al., 2002). The endpoint of the test was the total number of juveniles per test vessel at the end of the test; adult numbers were also registered.

2.4.2. Enchytraeids reproduction test

The reproduction tests were performed based on ISO 16387 (2004) guidelines with a few modifications. The test duration was 4 weeks instead of the 6 weeks indicated in ISO 16387 for the *E. albidus*, to accommodate the shorter reproductive cycle of *E. crypticus* (Kuperman et al., 2004). To assess the full dose-response relationships, concentrations series of azoxystrobin (10, 15, 20, 35, 50, 80, 120, 200, 300, 450, 650, 1000 mg a.i. kg^{-1} dw soil), chlorothalonil (5, 10, 20, 30, 40, 60, 90, 150, 200, 250, 300, and 500 mg a.i. kg^{-1} dw soil) and ethoprophos (20, 30, 45, 65, and 100 mg a.i. kg^{-1} dw soil) were selected. The test started with the introduction of ten adult enchytraeids with well developed clitella in glass test vessels, each containing approximately 20 g of dw soil and 50 mg of finely ground dry oats of food covered with soil particles. Two replicates per pesticide treatment were used and not the standard four replicates due to the high number of concentrations adopted in order to increase the robustness of the test and EC_{50} calculations. Four replicates were used for each of the control soils tested. An extra vessel without test organisms and food was prepared for each treatment concentration and used for pH (1 M KCl) and moisture determination at the end of the test (ISO 10390, 1994; ISO 16387, 2004). All replicas were weighed weekly for moisture loss replenishment and 25 mg of food added if needed. At the end of the test all enchytraeids in soil (adults and juveniles) were collected by transferring all test vessels content to a metal sieve (500 μm) placed in a bowl and filled with water so that the soil was submerged under the water. The organisms tended to stay at the surface of the soil and water and were collected with a plastic pipette. Each replicate group of organisms was fixed with alcohol and stained with Bengal red before counting. The measurement endpoint was the number of juveniles at the end of the test.

2.4.3. Earthworm reproduction test

The ecotoxicity tests followed the ISO 11268–2.2 (1998) guidelines. The following gradients of concentrations were selected to assess the full dose-response relationships for each pesticide: azoxystrobin (50, 100, 200, 300, and 500 mg a.i. kg^{-1} dw soil), chlorothalonil (5, 10, 20, 50, and 100 mg a.i. kg^{-1} dw soil) and ethoprophos (0.1, 0.3, 1, 3, and 12 mg a.i. kg^{-1} dw soil). Four replicates were used per test concentration and for each of the control soils tested. At the beginning of the test, cylindrical plastic vessels (500 ml) with perforated transparent lids to facilitate air circulation were filled with 500 g dw soil. Fifteen grams of moistened dry finely ground horse manure were added to each test vessel and ten earthworms, previously weighted, were placed on each of the test replicates. The groups of ten test organisms were paired randomly with each replicate and each group was weighted. The test vessels were weighed for weekly moisture loss and replenished if needed. After 4 weeks of exposure, living adults were removed by hand sorting and each replicate's living test organisms weighed for biomass determination. Mortality of adult individuals was assessed by counting the living organisms and any individuals not accounted for were considered dead. The soil and existing cocoons returned to the test vessels and 5 g of food added, and incubated for another 4 weeks to allow cocoon development. At the end of the test, juveniles were extracted from the test soil using a water bath kept at 50/60 °C and counted. The test endpoints were adult mortality and change of biomass after 4 weeks and number of juveniles produced after 8 weeks. Soil pH (1 M KCl) and moisture were determined at the beginning and at the end of the experiment for each concentration tested (ISO 10390, 1994; ISO 11268–2.2, 1998).

2.5. Calculations and statistical analyzes

Results were statistically analyzed according to EPS 1/RM/46 (2005) and using STATISTICA 7.0 (Stat Soft Inc, 2004).

Effect concentrations of 50% and 20% in reproduction tests and corresponding 95% confidence limits were calculated through concentration-response relationships using nonlinear regressions. The nonlinear regression model was selected in order to best describe the concentration–response trend with the help of scatter plots or line graphs for each experiment distribution and the proportion of variance accounted for (r^2). Model used was: (i) logistic: juveniles = $t/(1 + (\text{conc}/x)\hat{}b)$ where: t–y-intercept (control response); x—estimated EC value for the data set; b—a scale parameter (EPS 1/RM/46, 2005), with the estimation method of Levenberg–Marquardt. For the estimation of the EC_x values, the

Table 3
Average number of juveniles (±standard deviation) in the controls ($n = 4$) at the end of the terrestrial ecotoxicity tests conducted with the different soil organisms using natural and artificial soil.

Organism tested	Pesticide tested	Natural soil (sandy loam)	Artificial soil (OECD 10%)
Collembolans	Azoxystrobin	262 ± 79	250 ± 23
	Chlorothalonil	414 ± 29	130 ± 27
	Ethoprophos	317 ± 47	184 ± 53
Enchytraeids	Azoxystrobin	1204 ± 70	442 ± 163
	Chlorothalonil	1321 ± 143	729 ± 92
	Ethoprophos	1373 ± 543	1200 ± 100
Earthworms	Azoxystrobin	77 ± 3	36 ± 7
	Chlorothalonil	80 ± 11	34 ± 5
	Ethoprophos	75 ± 10	34 ± 5

normality for all test results was evaluated through a Q–Q plot of the residuals. The homogeneity of the variance was also evaluated after the analysis through a graphical distribution of the predicted versus the residual values.

On those tests where 4 replicates were used (Collembola tests with ethoprophos and Earthworms with all pesticides), NOEC (no observed effect concentration) and LOEC lowest observed effect concentration) values were estimated using a one way analysis of variance (ANOVA) followed by a Dunnett test. In this case normality of the distribution and homogeneity of the variance were tested using Kolmogorov–Smirnov (K–S) and Levene's tests, respectively. The same statistical procedure was performed to evaluate significant differences in earthworm mortality and biomass variations among chemical treatments and respective controls.

3. Results

3.1. Test soils

No pesticide residues were detected in the natural soil test which validates its use as a test soil for this study. The validity criteria of controls for each single species reproduction test were attained (collembolans: mortality of adults <20%; reproduction rate of >100 instars per vessel; coefficient of variation of reproduction <30%; enchytraeids: mortality of adults <20%; reproduction rate of >25 juveniles per vessel; coefficient of variation of reproduction <50%; and earthworms: mortality of adults ≤10%; reproduction rate of ≥30 juveniles per vessel; coefficient of variation of reproduction <30%). The pH and moisture content in the natural soil controls of the three terrestrial ecotoxicity tests were on average 4.71% and 20% at the start of the tests respectively, and increased by an average of 0.1 units and 0.11%, respectively, at the end of the test. Artificial control soil average pH decreased 0.1 units from the initial values of 5.63, and moisture content decreased 0.42% from the initial value of 29%. Generally the three test organisms reproduced twice as much in the natural soil control compared to the OECD artificial soil control (Table 3).

3.2. Exposure concentrations

The measured concentrations in the stock solutions of azoxystrobin and chlorothalonil were on average 97.8% and 93.5%, respectively, of those of the nominal stock solutions used for spiking the soil on the terrestrial tests. Since the nominal and measured concentrations did not differ substantially, no adjustments for recovery were made when calculating the toxicity endpoints. Ethoprophos concentration could not be measured due to laboratory technical difficulties but since identical work procedures were used, the risk of erroneous dosage in the present study was deemed minimal and the nominal concentrations were used for the toxicity endpoint assessment.

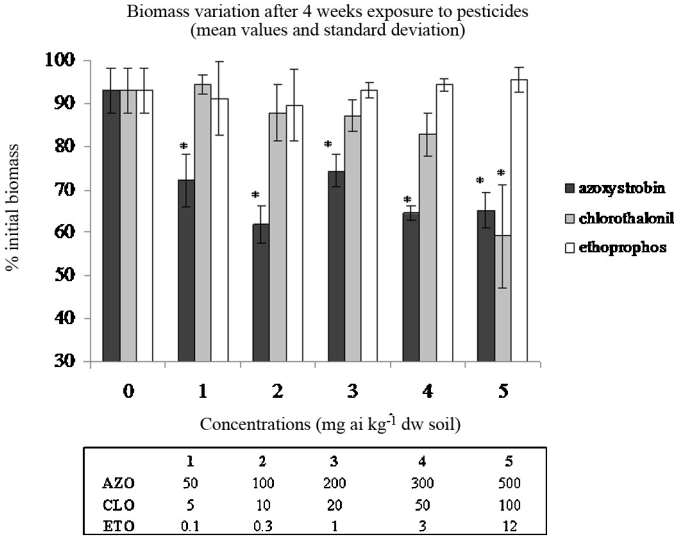

Fig. 1. Adult earthworm (*Eisenia andrei*) biomass variation after 4 weeks exposure to the tested pesticides (mean ±SD). * Significant differences with control ($p < 0.05$).

3.3. Assessment of pesticides effects to terrestrial organisms

In order to account for the differences in mass of each pesticide when comparing the results for the same organism between the pesticides, the active ingredient individual molar mass was used to transform the results values into mol of active ingredient (a.i.) per kg of dry weight of soil. This is the reason why results are shown in two types of units ('mg a.i. kg^{-1} dw soil' and 'mol a.i. kg^{-1} dw soil') in the text and Table 4.

3.3.1. Collembolans

Adult collembolans showed a maximum of 10% mortality at the higher concentration (1000 mg a.i. kg^{-1} dw soil) during the reproduction tests with azoxystrobin. The highest chlorothalonil exposure concentration resulted in 35% mortality effect on adult collembolans after 4 weeks of exposure (150 mg a.i. kg^{-1} dw soil). No adult collembolans were observed at the two highest ethoprophos concentrations (0.040 and 0.050 mg a.i. kg^{-1} dw soil) and a 65% mortality rate was found at 0.030 mg a.i. kg^{-1} dw soil. Ethoprofos had a significant effect on the reduction of juveniles at much lower concentrations (1000× less) compared to azoxystrobin and chlorothalonil test (Table 4), with an EC_{50} of 1.11×10^{-7} mol a.i. kg^{-1} dw soil. The EC_{50} of azoxystrobin (in mol a.i. kg^{-1} dw soil) was two times higher than that of chlorothalonil, herewith showing azoxystrobin to be less toxic for collembolans.

3.3.2. Enchytraeids

Enchytraeids were affected by the three chemicals at comparable concentrations (Table 4), with the EC_{50} values of 2.46 and 2.83×10^{-4} mol a.i. kg^{-1} dw soil for azoxystrobin and ethoprophos, respectively, which is approximately half of the chlorothalonil value of 4.25×10^{-4} mol a.i. kg^{-1} dw soil (Table 4).

3.3.3. Earthworms

The biomass of adult earthworms exposed to the control with natural soil for 4 weeks showed an average decrease of 7.0% compared to the initial biomass (Fig. 1). The exposure of *E. andrei* to azoxystrobin resulted in a significant weight

Table 4
Pesticides molecular mass and results of statistical analysis for sub-lethal effects on terrestrial organism reproduction for each pesticide.

Pesticide Mol mass (g mol^{-1})	Organism	EC_x (95% CI) (mg a.i. kg^{-1} dw soil)	EC_x (mol a.i. kg^{-1} dw soil)	Model r^2	NOEC-LOEC (mg a.i. kg^{-1} dw soil)	Normality homogeneity
AZO	*Folsomia candida*	EC_{50} = 92.0 (57.9–126.1)	$EC_{50} = 2.28 \times 10^{-4}$	0.88	–	–
		EC_{20} = 54.9 (23.0–86.9)	$EC_{20} = 1.36 \times 10^{-4}$	–	–	–
(403.4)	*Enchytraeus crypticus*	EC_{50} = 99.2 (73.3–125.7)	$EC_{50} = 2.46 \times 10^{-4}$	0.95	–	–
		EC_{20} = 42.6 (25.2–60.0)	$EC_{20} = 1.06 \times 10^{-4}$	–	–	–
	Eisenia andrei	EC_{50} = 42.0 (23.2–60.8)	$EC_{50} = 1.04 \times 10^{-4}$	0.96	<50	K–S $p > 0.20$
		EC_{20} = 12.2 (1.2–23.1)	$EC_{20} = 3.02 \times 10^{-5}$	–	50	Levene's $p = 0.07$
CLO	*Folsomia candida*	EC_{50} = 31.1 (24.7–37.5)	$EC_{50} = 1.17 \times 10^{-4}$	0.95	–	–
		EC_{20} = 18.2 (12.0–24.5)	$EC_{20} = 6.84 \times 10^{-5}$	–	–	–
(265.9)	*Enchytraeus crypticus*	EC_{50} = 112.9 (89.8–136.1)	$EC_{50} = 4.25 \times 10^{-4}$	0.955	–	–
		EC_{20} = 39.4 (25.6–53.3)	$EC_{20} = 1.48 \times 10^{-4}$	–	–	–
	Eisenia andrei	EC_{50} = 40.9 (30.1–51.7)	$EC_{50} = 1.54 \times 10^{-4}$	0.94	5	K–S $p > 0.20$
		EC_{20} = 20.8 (11.0–30.5)	$EC_{20} = 7.82 \times 10^{-5}$	–	10	Levene's $p = 0.09$
ETO	*Folsomia candida*	EC_{50} = 0.027 (0.024–0.031)	$EC_{50} = 1.11 \times 10^{-7}$	0.944	0.020	K–S $p > 0.10$
		EC_{20} = 0.021 (0.017–0.026)	$EC_{20} = 8.67 \times 10^{-8}$	–	0.030	Cochran C $p = 1.00$
(242.3)	*Enchytraeus crypticus*	EC_{50} = 68.5 (42.9–94.1)	$EC_{50} = 2.83 \times 10^{-4}$	0.77	–	–
		EC_{20} = 41.2 (17.2–65.2)	$EC_{20} = 1.70 \times 10^{-4}$	–	–	–
	Eisenia andrei	EC_{50} = 8.3 (3.6–13.0)	$EC_{50} = 3.43 \times 10^{-5}$	0.76	3	K–S $p > 0.20$
		EC_{20} = 3.5 (0–7.1)	$EC_{20} = 1.44 \times 10^{-5}$	–	12	Levene's $p = 0.12$

Mol mass—molecular mass (MacBean, 2012); AZO—azoxystrobin, CLO—chlorothalonil, ETO—ethoprophos; CI—Confidence interval; p—probability value.

loss (Dunnett test $p < 0.05$) throughout the concentration gradient (Fig. 1). A 7.7% adult mortality was observed only at the higher concentration ($LC_{50} > 500$ mg a.i. kg^{-1} dw soil). Exposure to chlorothalonil resulted in a weight loss gradient (5.5–40.9%) with increasing pesticide concentration (Fig. 1) with only significant values for the highest concentration of 100 mg a.i. kg^{-1} dw soil. This biomass decrease was accompanied by a mortality rate of 59.0% only at the highest concentration resulting in a LC_{50} for adults of approximately 95.0 mg a.i. kg^{-1} dw soil. No adult earthworm mortality was observed after 4 weeks exposure to ethoprophos ($LC_{50} > 12$ mg a.i. kg^{-1} dw soil), and a slight (but not significant) gain of weight was determined (91.1 to 95.5% of initial biomass) along the concentration gradient (Fig. 1). The highest toxicity on earthworms' reproduction was found for ethoprophos resulting in an EC_{50} of 3.43×10^{-5} mol a.i. kg^{-1} dw soil (Table 4). The inhibition of juvenile production by earthworms under azoxystrobin and chlorothalonil exposure resulted in similar EC_{50} toxicity values (1.04×10^{-4} mol and 1.54×10^{-4} mol a.i. kg^{-1} dw soil, respectively). Nevertheless, EC_{20} values differed between these pesticides with azoxystrobin being more toxic (Table 4). A significant reduction in juvenile numbers (Dunnett test $p < 0.05$) was observed for all the three pesticides allowing LOEC and NOEC calculations (Table 4). However, in azoxystrobin all tested concentrations were significantly different from the control ($p < 0.005$) resulting in effects on earthworms, which did not allow for a NOEC value to be attained (NOEC < 50 mg a.i. kg^{-1} dw soil).

4. Discussion

The study focused on evaluating the effects on the reproduction of non-target soil organisms for three commonly used pesticides in irrigated crops using a Mediterranean natural soil. All the test organisms presented different toxicity responses to the tested pesticides (Table 4). This could be associated with the processes of chemical uptake by the organisms and the different types of toxic action of the pesticides (Frampton et al., 2006). Uptake of organic contaminants by terrestrial organisms is intimately associated with the soil pore water which is in general the dominant pathway (EFSA, 2009b; Styrishave et al., 2008). Soft bodied soil organisms such earthworms and enchytraeids take pesticides up either through passive diffusion from pore water through the skin or by ingestion together with soil particles (De Silva et al., 2009). Hard-bodied soil organisms as collembolans take oxygen and water through specialized organs also from the soil pore water (EFSA, 2009b). In addition, pesticides bioavailability through the soil pore water can be influenced by soil properties such as organic matter (OM) and clay content (increase of OM and clay) that relates to sorption restraining the pesticide molecules in a form that is not available for organism uptake (EFSA, 2009b; Kuperman et al., 2006; Van Gestel, 2012). This fact has been reported by several authors for soil-dwelling organisms such as enchytraeids and earthworms, for a number of compounds: organochlorine and carbamate insecticides, benzimidazole, and polychlorinated fungicides, among others (Amorim et al., 2002a,b; De Silva et al., 2009; Lanno et al., 2004; EFSA, 2009a; Patakioutas and Albanis, 2002).

4.1. Effects of azoxystrobin on soil biota

In spite of the low solubility of azoxystrobin in water as active ingredient (Table 1), its partition to the soil pore water may be expected due to its formulation product being water soluble as concentrated suspension. Azoxystrobin is expected to have low environmental toxicity to earthworms and terrestrial arthropods due to its chemical group characteristics (strobilurin), as to be relatively readily degraded in the environment causing little potential for chronic exposure (Bartlett et al., 2002). However, the present study revealed a higher sub-lethal effect response of azoxystrobin to earthworms (EC_{50} of 42.0 mg a.i. kg^{-1} dw natural soil) compared to collembolans and enchytraeids (Table 4). Although a significant biomass decrease was observed on earthworms at the lowest concentration (50 mg a.i. kg^{-1} dw soil) (Fig. 1), resulting in effects on their reproduction, only 7.7% mortality was observed at the highest concentration. Even though biomass and mortality are always registered together as test endpoints, Potter et al. (1994) verified that the loss in biomass was independent of the lethal effects of chemicals. The observed low lethal toxicity to earthworms with natural soil differ greatly from the reported results with OECD artificial soil tests showing LC_{50} values of 283 mg a.i. kg^{-1} soil (EFSA, 2010b) and 327.4 mg a.i. kg^{-1} soil (Wang et al., 2012). Nevertheless, the fact that the NOEC for earthworms test was not attained with the lowest concentration tested (50 mg a.i. kg^{-1} soil) is in agreement with the reported NOEC of 20 mg a.i. kg^{-1} soil for *E. fetida* (FOOTPRINT, 2012).

4.2. Effects of chlorothalonil on soil biota

Chlorothalonil is not expected to partition to the soil pore water as active ingredient due to its low solubility in water and high

sorption constant facilitating adsorption to soil particles (Table 1). However, by using the concentrated suspension formulation, a movement to the soil pore water may be expected due to its water solubility. If present in the water fraction of the soil (soil pore water) the pesticide can be bioavailable for uptake by the soil organisms (Styrishave et al., 2008). In terms of sub-lethal effects of chlorothalonil, collembolans were the most sensitive taxa followed by the earthworms (Table 4). The enchytraeids were the least sensitive with an EC_{50} ratio of almost 3:1 of the other two organisms. This low sensitivity of enchytraeids towards chlorothalonil has also been reported for other pesticides such as a polychlorinated insecticide in specific and other fungicides of the same chemical group, and insecticides in a broader evaluation (Bezchlebová et al., 2007; Daam et al., 2011b; Frampton et al., 2006). The NOEC for earthworms of 5 mg a.i. kg^{-1} dw soil is in agreement with the reported NOEC value of 1.65 mg a.i. kg^{-1} soil from tests with the same formulation (500 g a.i. L^{-1} SC), and a 5% OM OECD soil (EC, 2006), which is similar to the organic matter content of the natural soil used in this study (Table 2). The 59% mortality of adult earthworms observed at the highest tested concentration, of 100 mg a.i. kg^{-1} dw soil after 4 weeks exposure to chlorothalonil, occurs at a concentration which is two times lower than the reported earthworms acute test effect concentration (LC_{50}) of 268.5 mg a.i. kg^{-1} soil (EC, 2006). Although this value is attained from a test with artificial soil with an organic matter content of 5% (EC, 2006) similar to the tested natural soil, this difference in the lethal effects results may be due to other factors associated to the natural soil such as pH and clay content influencing pesticide availability (EFSA, 2009b).

4.3. Effects of ethoprophos on soil biota

The insecticide ethoprophos by having high solubility in water and low sorption coefficient (Table 1) could be expected to be present in the soil pore water. This movement to the water compartment could also be expected due to the pesticide leaching capacity (GUS; Table 1), and as such, becoming available for uptake by the soil organisms (Styrishave et al., 2008). Collembolans were the most affected by ethoprophos with a low EC_{50} of 0.027 mg a.i. kg^{-1} dw soil, which would be expected from the type of toxic action as an insecticide towards arthropods (Daam et al., 2011b; Frampton et al., 2006). The earthworms presented the second lowest EC_{50} value of 8.3 mg a.i. kg^{-1}dw soil and the enchytraeids were the least sensitive with an EC_{50} value more than 8 times higher than the collembolan's (EC_{50} = 68.5 mg a.i. kg^{-1} dw soil). In spite of such, no mortality effects on adult earthworms were observed and a slight gain in weight was observed. This test results are in congruence with reported values of LC_{50} 39.6 mg a.i. kg^{-1} dw soil (EFSA, 2006) since the maximum tested concentration during this study was 12 mg a.i. kg^{-1} dw soil. The observed sub-lethal effects on cocoon production and viability may be a consequence of the pesticide intake by the adults that even at low dosages can cause adverse effects during long term exposures. The reported NOEC value of <1.67 mg a.i. kg^{-1} dw soil (EFSA, 2006) from a test with artificial soil is lower than the study test results, which shows that different soils may cause different toxicity results, as referred above.

4.4. Sensitivity of the three invertebrate arthropods to the pesticides

The higher sensitivity of collembolans (*F. candida*) observed in this study as compared with the other organisms for two pesticides, the fungicide chlorothalonil and the insecticide ethoprophos, has been found for a wide range of pesticides with different type of toxic action, suggesting that the earthworms are not always the most sensitive species (Bezchlebová et al., 2007; Daam et al., 2011b; Frampton et al., 2006). However, care should be taken when making generalizations of effects of pesticides within the same chemical group where significantly different toxicities may occur in a single group of soil organisms (e.g. effects on earthworms among the strobilurin group (Wang et al., 2012)). Enchytraeids were mainly the least sensitive of the three species tested for reproductive effects. Although reports have shown that they are generally less sensitive than lumbricidae when assessing acute data such as LC_{50} (EFSA, 2009b), the results obtained in our study contradict the results reported by Römbke and Moser (2002) which report a similar sensitivity of the two organism groups regarding reproductive effects in different soil substrates (artificial and natural). These differences in long-term exposure tests reflect the difficulty in grouping pesticides effects on non-target organisms. This emphasizes the need to include arthropods and other annelids as relevant organisms in the first tier of pesticide environmental risk assessment in order to better represent and protect the terrestrial environment against the wide existing group of pesticides (Frampton et al., 2006).

5. Conclusion

Results showed that the use of only the earthworm as a key species for the first tier terrestrial ERA of pesticides may not be enough to ascertain a significant protection level of the terrestrial ecosystem by not being the most sensitive organisms, especially for the tested insecticide. Moreover, the use of natural soil may lead to differences in toxicity values compared with OECD referenced values. This illustrates the importance of creating realistic scenarios under the first tier ERA, since artificial soils may not allow a realistic approach for the evaluation of pesticide toxicity. Natural soil variations are accounted for in the Guidance Document on Terrestrial Ecotoxicology (SANCO, 2002) under a risk assessment for earthworms. However, with the revision on the data requirements for active substances (EU, 2013), and the division of the EU territory into three zones (north, central, and south) by the new regulation concerning the placing of plant protection products on the market (EC, 2009), understanding the different behaviour of pesticides and their availability in different soils types becomes of great importance.

Acknowledgements

This study was partly sponsored by Fundação para a Ciência e Tecnologia, Portugal, through a Ph.D. grant (SFRH/BD/42306/2007). We would like to thank Cátia Silva and all the colleagues at the Soil Ecology and Ecotoxicology Laboratory of Life Sciences Department of Coimbra University for their knowledge and technical support of this study.

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