Chemosphere 90 (2013) 2674–2682

Contents lists available at SciVerse ScienceDirect

Chemosphere

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

Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions

Paulo Roger L. Alves [a,c,*], Elke J.B.N. Cardoso [a], Alexandre M. Martines [a], José Paulo Sousa [b], Amarildo Pasini [c]

[a] Dep. of Soil Science, University of São Paulo, Pádua Dias, 11, 13416-260 Piracicaba, Brazil
[b] IMAR-CMA, Dep. of Zoology, University of Coimbra, P3004-517 Coimbra, Portugal
[c] Dep. of Agronomy, Universidade Estadual de Londrina, 86051-990 Londrina, Brazil

HIGHLIGHTS

- ▶ The toxicity of seed dressing pesticides was tested on earthworms.
- ▶ Lower-tier laboratory tests were performed in tropical conditions.
- ▶ Only the pesticide with imidacloprid caused mortality in *Eisenia andrei*.
- ▶ All the tested pesticides showed negative effects in the chronic toxicity test.
- ▶ Avoidance tests were the most sensitive for the substances investigated in the study.

ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form 15 September 2012
Accepted 24 November 2012
Available online 21 December 2012

Keywords:
Seed treatment
Lower-tier tests
Eisenia andrei
Environmental risk assessment
Soil fauna
Soil ecotoxicology

ABSTRACT

Ecotoxicological laboratory tests (lower-tier tests) are fundamental tools for assessing the toxicity of pesticides to soil organisms. In this study, using these tests under tropical conditions, we quantified the impact of the insecticides imidacloprid, fipronil, and thiametoxam, and the fungicides captan and carboxin + thiram, all of which are used in the chemical treatment of crop seeds, on the survival, reproduction, and behavior of *Eisenia andrei* (Oligochaeta). With the exception of imidacloprid, none of the pesticides tested caused mortality in *E. andrei* in artificial soils. The LC_{50} of imidacloprid was estimated as 25.53 mg active ingredient kg^{-1} of dry soil. Earthworm reproduction rates were reduced by imidacloprid (EC_{50} = 4.07 mg kg^{-1}), fipronil (EC_{20} = 23.16 mg kg^{-1}), carboxin + thiram (EC_{50} = 56.38 mg kg^{-1}), captan (EC_{50} = 334.84 mg kg^{-1}), and thiametoxam (EC_{50} = 791.99 mg kg^{-1}). Avoidance behavior was observed in the presence of imidacloprid (AC_{50} = 0.11 mg kg^{-1}), captan (AC_{50} = 33.54 mg kg^{-1}), carboxin + thiram (AC_{50} = 60.32 mg kg^{-1}), and thiametoxam (AC_{50} = >20 mg kg^{-1}). Earthworms showed a preference for soils with the insecticide fipronil. Imidacloprid was the most toxic of the substances tested for *E. andrei*. The avoidance test was the most sensitive test for most pesticides studied, but results varied between pesticides. These results offer new insights on the toxicity of pesticides used to treat seeds in tropical regions. However, they should be complemented with higher-tier tests in order to reduce the uncertainties in risk assessment.

1. Introduction

Treating seeds with pesticides is a practice used in Integrated Pest Management (IPM) that helps prevent soil-borne pests and pathologies and reduces losses at the beginning of the crop cycle (Munkvold et al., 2006). Most of the world's cereal crops grow from seeds treated with insecticides and fungicides (Brühl et al., 2011). The use of these and other agricultural defenses is increasing globally, and in 2010 Brazilian farmers spent more than US$ 1.5 billion importing pesticides (FAO, 2012).

Although the pesticides used to treat seeds and for other agricultural applications are fundamental for maintaining high levels of food production, there are serious concerns about these substances' potential for pollution. In the case of soil pollution, one focus of study has been the effect pesticides have on the soil fauna

* Corresponding author at: Dep. of Soil Science, University of São Paulo, Av. Pádua Dias, No. 11, 13416-260 Piracicaba, São Paulo, Brazil. Tel.: +55 19 3417 2142(O); fax: +55 19 3417 2110.

E-mail address: pauloroger@usp.br (P.R.L. Alves).

0045-6535/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.chemosphere.2012.11.046

and the implications for the many biological processes that involve the soil fauna (Cardoso and Alves, 2012). In Europe, specific laws (EC1107/2009) regulate the use of pesticides in soils (EC, 2009) and prescribe ecotoxicological and other tests to assess the effects of pesticides on soil invertebrates (Jänsch et al., 2006). In Brazil, a recently passed law on soil quality management requires that pesticide risk be assessed following methods that are recognized by internationally recognized norms (CONAMA, 2009).

Ecotoxicological laboratory tests represent the worst-case scenario, and are considered a preliminary step (the lower tier) in assessments of environmental risk. Because they yield relatively quick results, these tests can quantify the risks to fauna posed by the use of certain substances in the soils of a given terrestrial ecosystem. In the case of pesticides, several studies have described the effects on earthworms of various classes of these chemicals (Frampton et al., 2006) and have accumulated a database that allows one to weigh the toxicity of a given active ingredient (a.i.) against its benefits. However, toxicity studies on soil fauna (i.e., lower-tier tests) have not yet been carried out for several classes of pesticides (Jänsch et al., 2006). Such tests are also required to implement legislation that can effectively regulate plant protection products (Jänsch et al., 2006). Under tropical conditions, however, the number of studies that have reported the impacts of pesticides on earthworms remains small (Garcia et al., 2008; De Silva et al., 2010; García-Santos and Keller-Forrer, 2011; Nunes and Espíndola, 2012).

Among the lower-tier earthworm tests, the two most common are acute toxicity tests, which are designed to detect qualitative effects and determine lethality, and chronic toxicity (reproduction) tests, which are capable of detecting more subtle effects, such as retarded development, reduced fertility, and teratogenic effects, and can also reveal qualitative and quantitative changes in earthworm populations even where mortality does not occur (Edwards, 2004). Like reproduction tests, avoidance tests are sublethal assessments based on earthworm behavior. Among the advantages offered by these tests are the short time they require (48 h), low cost, and a sometimes higher sensitivity compared to other toxicity tests (García-Santos and Keller-Forrer, 2011).

Gaucho® (Bayer AG), a neonicotinoid-class insecticide whose a.i. is imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine], is a commercially available product for treating seeds. While we did not find reports of effects of this specific commercial formulation on earthworms, the a.i.'s impact on oligochaetes has been described (Luo et al., 1999; Capowiez and Berard, 2006; Kreutzweiser et al., 2008; Gomez-Eyles et al., 2009). The same is true of the insecticides Standak® (BASF), whose a.i. is fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazol-3-carbonitrile), a phenylpyrazol (Mostert et al., 2002); and Cruiser® (Syngenta), whose a.i. is thiametoxam (3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5] oxadiazinan-4-ylidene-N-nitroamine), also a neonicotinoid (NRA, 2001; EC, 2007). By contrast, acute toxicity effects on earthworms have been described for the fungicides Captan® (Milenia Agrosciences), whose a.i. is captan (N-(trichloromethyltio)cyclohex-4-ene-1,2-dicarboximide), in the dicarboximide class; and Vitavax® (Chemtura), whose a.i. are carboxin (5,6-dihydro-2-methyl-1,4-oxatiina-3-carboxanilide) + thiram (tetramethylthiuram disulphide), in the dithiocarbamate and carboxanilide classes, respectively (Anton et al., 1990; EFSA, 2010). These fungicides are still widely used to treat seeds.

The objective of this study was to characterize, via lower-tier ecotoxicological tests, the effects of varying concentrations of three insecticides and two fungicides used to treat seeds on the survival, reproduction, and behavior of the earthworm *Eisenia andrei* under tropical climatic conditions in the laboratory.

2. Materials and methods

2.1. Study organisms and test conditions

For the ecotoxicological assays we reared European earthworms of the species *E. andrei* (Lumbricidae). Methods were adapted from International Organization for Standardization (ISO) norm 11268-2 (ISO, 1998). The substrate used to rear the earthworms was a mixture of dried and sifted horse manure, powdered coconut husk, and fine sand (>50% of grains measuring 0.05–0.2 mm), in the proportion 2:1:0.1 by dry weight (d.w.), respectively. All animals in the mixture were killed by a defaunation process (Pesaro et al., 2003) consisting of three 48-h cycles of freezing and thawing. Acidity (pH) of the mixture was corrected with $CaCO_3$ to 6.5 ± 0.5.

The worms were reared and all bioassays carried out in a climate-controlled room with a higher temperature of 23 ± 2 °C and a 12 h photoperiod. Avoidance tests were carried out in the dark. Twenty-four hours before the bioassays were started, worms were acclimated to the untreated test soil. Only adult (clitellate) worms with an individual body weight of 300–600 mg were used in the study.

2.2. Artificial soil and pollutants

Ecotoxicological assays were carried out in Tropical Artificial Soil (TAS), an adaptation of Garcia (2004) of artificial OECD (Organisation for Economic Co-operation and Development) soil (OECD, 1984). This soil was a mixture of fine sand (>50% of grains measuring 0.05–0.2 mm), kaolinitic clay (powdered kaolin), and powdered coconut husks, in a proportion of 70:20:10 d.w., respectively. After the TAS was mixed and homogenized, its acidity was corrected where necessary with $CaCO_3$ to 6.0 ± 0.5. We also determined the water holding capacity (WHC) of the TAS, following ISO (1998), and immediately before the tests were begun corrected soil humidity to a mean value of 60% WHC, using water for the control and diluted solutions for the treatments. Soil pH was determined via 1 mol L^{-1} KCl (1:5 w/w) at the start and end of each bioassay.

We selected five pesticides that are commonly used in agriculture: the insecticides Gaucho®, Standak®, and Cruiser®, and the fungicides Captan® and Vitavax®. All have different a.i. (Table 1). Before tests were begun, all pesticides were diluted and homogenized in deionized water. Pesticides were applied to the soil during the correction of soil humidity, as described above, in such a way that the solutions/suspensions of the pesticides were evenly distributed throughout the soil. Only deionized water was added to the control.

We estimated the volume of each pesticide exposed to the soil in the commercially used doses. These values were obtained by multiplying the volume of each pesticide recommended per kg of soybean seeds by the weight of seeds used per hectare (ha) (EMBRAPA, 1999), an extrapolation which yielded the amount of pesticide applied per ha. Assuming a soil density of 1 g cm^{-3} and a

Table 1
A description of the active ingredients (a.i.) of the studied pesticides and their predicted environmental concentrations (PECs) in commercial doses for soybean crops.

Commercial name	a.i. name	a.i. content (g L^{-1})	PEC (mg of a.i. kg^{-1} dry soil)
Gaucho® 600 FS	Imidacloprid	600	0.23
Standak® 250 SC	Fipronil	250	0.096
Cruiser® 350 FS	Thiamtoxam	350	0.201
Captan® 480 SC	Captan	480	0.23
Vitavax® 200 SC	Carboxin + thiram	200	0.115

depth of incorporation in the soil profile of 0–5 cm, we obtained the predicted environmental concentrations (PECs) of each a.i. (mg kg^{-1} soil d.w.) potentially present in the soil during a single soybean planting cycle (Table 1).

The concentrations of each pesticide used in the laboratory tests were determined through range finding tests. We started with an acute toxicity test using increasing concentrations, up to the limit of 1000 mg a.i. per kg dry soil (mg kg^{-1} soil d.w.). Based on the results of these tests we selected a range of concentrations for the definitive tests of acute toxicity. The chronic toxicity bioassays used sublethal concentrations based on the definitive mortality tests. The avoidance tests used a different set of sublethal concentrations, lower than those that showed effects in the chronic toxicity test (Table 2).

2.3. Acute toxicity test

Lethal effects of each pesticide on *E. andrei* were assessed via acute toxicity tests, following OECD (2004). To circular plastic containers with a diameter of 12.5 cm and a height of 9.5 cm we added approximately 700 g of artificial soil, wetted with water or a pesticide solution, such that each container contained soil to a depth of 5–7 cm. The TAS was treated with five concentrations of each pesticide (Table 2), each with four replicates. Immediately before the start of the test, worms were washed and weighed individually and 10 individuals were placed in each test container, which were closed with perforated lids. The worms were fed horse manure once a week during the 14 d of the test. On the last day of the bioassay, worms were removed from the containers. Individuals that did not respond to mechanical stimulation of the anterior portion of the body were recorded as dead. Live worms were washed and weighed, and the difference between starting and ending body weight was calculated.

2.4. Chronic toxicity test

The effects of each pesticide on earthworm reproduction were assessed using the ISO:11268-2 chronic toxicity test (ISO, 1998). Bioassays were installed as in the acute toxicity tests, and differed only in duration and assessment method. The TAS was treated with sublethal concentrations of each pesticide (Table 2), with four replicates per treatment. Bioassays were run for 56 d. After 28 d all adult worms were removed and weighed, and for the next 28 d only the soils, juvenile worms, and cocoons remained in the containers. On day 56 the containers were immersed in warm water (60 ± 5 °C) for 1 h, and once the juveniles had emerged on the soil surface we recorded the number of individuals generated during the period in which the adults were present. Worms were fed with horse manure once a week during the 56-d bioassay.

2.5. Avoidance test

Avoidance tests were carried out following the recommendations of ISO protocol 17512-1 (ISO, 2008). Each pesticide was tested at varying concentrations (Table 2) with five replicates each. Rectangular plastic boxes (23.3 cm long, 16.7 cm wide, 7.7 cm high) were divided into two equal compartments with a plastic divider. To each compartment we added 900 g of wetted soil, with one compartment of each container receiving treated soil and the other a control. Immediately thereafter, the plastic divider was removed and 10 adult *E. andrei* were placed on the line separating the two compartments of the containers. Containers were closed with perforated lids. The worms received no food during the test. After 48 h the plastic divider was inserted again and the soil in both compartments removed. We recorded the number of individuals present in each treatment (polluted/control). Worms that were along the dividing line between the two compartments were counted as 0.5 for each compartment.

To guarantee that the earthworms were distributed homogeneously throughout the two compartments of each container, and were not influenced by the surroundings or some other factor, a double-control test was carried out. Uncontaminated soil was placed in both compartments, in five replicates (ISO, 2008). While the procedures in this test were the same as described above for treated soils, the expected mean distribution of worms in a given compartment was 40–60%.

2.6. Data analyses

Results of the acute toxicity and avoidance tests were calculated as percentages. In the chronic toxicity test, we calculated the means of body weight difference and number of juveniles. Percent avoidance was calculated following Amorim et al. (2005), using this equation:

$$\%\text{avoidance} = [(C - T)/N] \times 100$$

where C is the number of worms in the control soil, T is the number of worms in the polluted soil, and N is the total number of worms at the start of the test. A positive percentage indicates avoidance of the polluted soil, a zero indicates no avoidance, and a negative percentage indicates an attraction for the pesticide-treated TAS (Amorim et al. 2005).

Analysis of variance (ANOVA) was used to test for significant differences between treatment means (i.e., different concentrations of pesticide) for the acute and chronic toxicity tests ($P < 0.05$). Where significant differences were detected, treatment means were compared to the control using Dunnett's test, in the SAS 9.2 software program. In this way we established NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) values for the toxicity tests.

Significant responses of the avoidance tests were analyzed with Fisher's exact test, using the two-tailed test for the double-control and the one-tailed test for the polluted soils, after Natal-da-Luz et al. (2008). Based on these results, NOEC and LOEC values for the avoidance tests were determined. We also carried out probit analyses using PriProbit 1.63 software, to obtain AC_{50} (avoidance concentration of 50%) values for the avoidance tests, and LC_{50} (lethal concentration of 50%) values for the acute toxicity tests.

Table 2
Concentrations of the active ingredients (a.i.) of each studied pesticide used in the acute toxicity, chronic toxicity, and avoidance tests.

Active ingredient	Tested concentration (mg kg^{-1} dry soil of a.i.)		
	Acute test	Chronic test	Avoidance test
Imidacloprid	6.25; 12.5; 25; 50; 100	0.75; 1.25; 2.50; 5; 10; 20	0.125; 0.25; 0.5; 1; 2
Fipronil	62.5; 125; 250; 500; 1000	0.1; 1; 62.5; 125; 250; 500; 1000	0.1; 1.25; 2.5; 5; 10
Thiametoxam	62.5; 125; 250; 500; 1000	1.47; 62.5; 125; 250; 500; 1000	1.25; 2.5; 5; 10; 20
Captan	62.5; 125; 250; 500; 1000	0.84; 1.68; 62.5; 125; 250; 500; 1000	0.625; 1.87; 5.62; 16.87; 50.62
Carboxin + thiram	62.5; 125; 250; 500; 1000	0.42; 62.5; 125; 250; 500; 1000	0.313; 1.25; 5; 20; 80

To estimate EC_{50}/EC_{20} values for the tests of chronic toxicity (i.e., pesticide concentrations which reduce worm reproduction by 50%/20%), we used non-linear regressions with pre-defined models in the Statistica 7.0 software program.

3. Results

3.1. Test validation

In the controls of the acute toxicity tests, and in all treatments of the chronic toxicity tests, mortality of adult *E. andrei* was below 10%, satisfying the ISO (1998) requirements of bioassay validation. In the chronic toxicity test the control showed a mean of >30 juvenile *E. andrei* individuals and a coefficient of variation (CV) of $\leqslant$30% per experimental unit. In the avoidance tests the number of dead and missing worms was $\leqslant$10%, and in the double control test there was a mean distribution of 40–60% of the organisms in each compartment of the container (ISO, 2008).

3.2. Acute toxicity responses

With the exception of imidacloprid, none of the studied pesticides were lethal to *E. andrei*. The mortality observed after exposure to the neonicotinoid only occurred at concentrations of 25 mg kg^{-1} (soil d.w.) or higher (Table 3), with an estimated LC_{50} of 25.53 (mg kg^{-1} soil d.w.). For the same LOEC at which mortality and reduced reproductive rates were observed for imidacloprid (Table 3), surviving worms also had reduced body weight, and in some cases, morphological changes that later resulted in death.

3.3. Chronic toxicity responses

Adult *E. andrei* produced fewer juveniles in all treatments (Fig. 1). Imidacloprid was the most toxic, given that the lowest concentration that significantly reduced reproduction (LOEC = 0.75 mg kg^{-1} soil d.w.) was the least of all the pesticides, and close to the PEC (Table 1). After imidacloprid, the pesticides that required the lowest concentrations to achieve significant toxicity, in decreasing order of toxicity, were the fungicides carboxin + thiram and captan, and the insecticides fipronil and thiametoxam. Thiametoxam was the least toxic, and only showed effects above concentrations of 500 mg kg^{-1} (soil d.w.) (Table 3).

With the exception of the insecticide imidacloprid, earthworms showed no reduction in body weight after 28 d of exposure to pesticides, compared to controls (Fig. 2). Earthworms in TAS treated with fipronil at the lowest concentration tested (Table 2) lost less body weight than worms in the control (Fig. 2).

3.4. Avoidance responses

Worms avoided TAS treated with the insecticides imidacloprid and thiametoxam, as well as those treated with the fungicides captan and carboxin + thiram (Fig. 3), allowing us to determine AC_{50} values for these pesticides (Table 3). For the last three, at the lowest concentrations (Table 2), more worms were found in the polluted compartments than in the control compartments, and apparently preferred soils with low concentrations of pesticides to those with deionized water (Fig. 3). However, TAS treated with higher concentrations of these three pesticides was avoided by the worms. The TAS treated with fipronil was preferred by the worms at all concentrations tested (Fig. 3).

4. Discussion

4.1. Acute toxicity test and biomass changes

In this study *E. andrei* mortality and body weight reduction (Fig. 2) effects only occurred in individuals exposed to TAS treated with concentrations of imidacloprid (LC_{50} = 25.53 mg kg^{-1} soil d.w., $LOEC_{biomass14d}$ 25 mg kg^{-1} soil d.w., and $LOEC_{biomass28d}$ 20 mg kg^{-1} soil d.w). Gomez-Eyles et al. (2009) observed similar effects in *E. fetida* when using concentrations about 10 times lower (LC_{50} = 2.36 mg kg^{-1} soil d.w. and $LOEC_{biomass28d}$ 1.91 mg kg^{-1} soil d.w.) following the application of the same active ingredient (98% pure), and by Kreutzweiser et al. (2008) in *E. fetida* (LC_{50} = 25.00 mg kg^{-1} soil d.w. and $LOEC_{biomass35d}$ 14 mg kg^{-1} soil d.w.) and *Dendrobaena octaedra* (LC_{50} = 5.7 mg kg^{-1} soil d.w. and $LOEC_{biomass35d}$ 3 mg kg^{-1} soil d.w.), after 35 d of exposure to Merit Solupak® (imidacloprid WP 750 g kg^{-1}). The differences between the results reported in the literature and those of our study are small, and mostly attributable to different substrate composition

Table 3
Toxicological parameters (NOEC, LOEC, LC_{50}, EC_{50}, EC_{20} and AC_{50}) calculated based on observations of *E. andrei* worms exposed to the five studied pesticides (values in mg kg^{-1} soil d.w.).

Test	Parameter	Active ingredient (mg kg^{-1} soil d.w.)				
		Imidacloprid	Fipronil	Thiametoxam	Captan	Carboxin + thiram
Acute	NOEC	12.50	1.000	1.000	1.000	1.000
	LOEC	25.00	>1000	>1000	>1000	>1000
	Upper limits (95%)	26.69	n.i.	n.i.	n.i.	n.i.
	LC_{50}	25.53	>1000	>1000	>1000	>1000
	Lower limits (95%)	24.44	n.i.	n.i.	n.i.	n.i.
Chronic	NOEC	<0.75	1.00	250	100	12.50
	LOEC	0.75	62.50	500	200	25.00
	Upper limits (95%)	5.72	n.i.	1238.97	432.17	79.82
	EC_{50}	4.07	>1000	791.99	334.84	56.38
	Lower limits (95%)	2.42	n.i.	345	237.51	32.95
	Upper limits (95%)	1.84	61.81	142.23	260.69	32.16
	EC_{20}	1.31	23.16	79.31	170.84	16.88
	Lower limits (95%)	0.78	15.49	16.39	80.98	1.60
Avoidance	NOEC	<10	10	2.5	16.88	20
	LOEC	0.13	>10	5	50.63	80
	Upper limits (95%)	n.i.	n.i.	n.i.	n.i.	n.i.
	AC_{50}	0.11	>10	>20	33.54	60.32
	Lower limits (95%)	n.i.	n.i.	n.i.	n.i.	n.i.

n.i., Value not estimated.

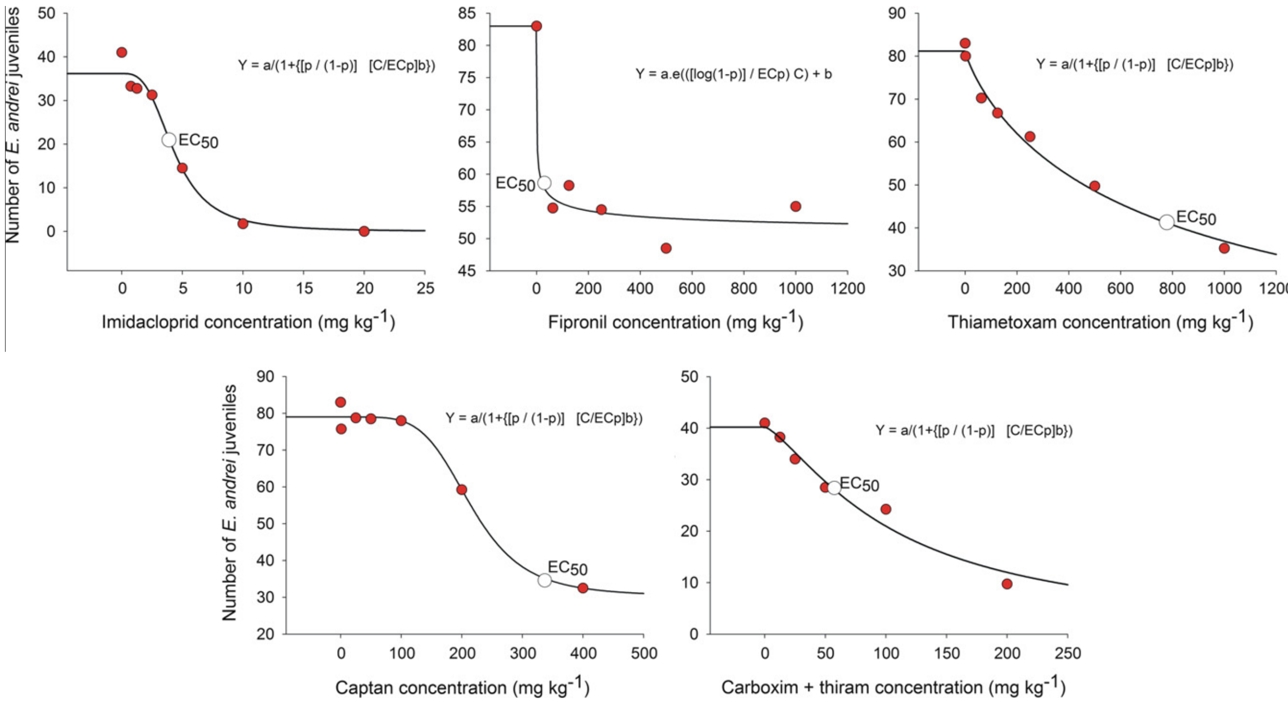

Fig. 1. Mean number of juvenile *E. andrei* earthworms found in TAS with varying concentrations of the five studied pesticides.

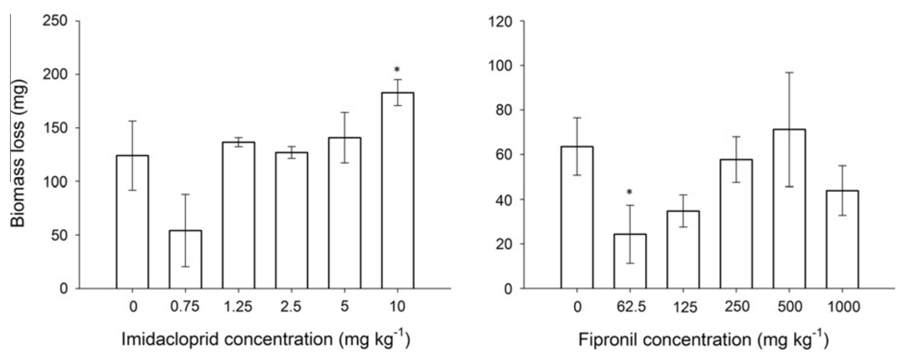

Fig. 2. Reduction in biomass (mg) of *E. andrei* in TAS treated with varying concentrations of imidacloprid and fipronil (28 d of exposure). *Means differ significantly from control (Dunnett's test, $p < 0.05$).

(Gomez-Eyles et al., 2009) and differing worm species (Kreutzweiser et al., 2008). It is known that LC_{50} values vary with organic matter, texture, pH, temperature, humidity, time of exposure, and other factors (Belfroid et al., 1993; Kula and Larink, 1997). Although the LC_{50} in this study and in the literature is 10–100 times higher than the PEC (Table 1), imidacloprid should not be classified as harmless for survival of *E. andrei*, based only in laboratory acute toxicity tests with artificial soil.

Reduced body weight may reflect reduced feeding by the worms, as seen in other studies (Capowiez and Berard, 2006; Gomez-Eyles et al., 2009). Those authors noted that worms excavate less when exposed to imidacloprid, which means that they feed less and have fewer intestinal contents. However, Kreutzweiser et al. (2008) noted in their experiments that worm feeding was similar in treated and control soils, and attributed the reduction in biomass to physiological changes in the worms. In this context, Luo et al. (1999) had earlier shown that at low concentrations of this a.i. (0.1 mg L^{-1} during 4 h in artificial soil) there was reduced activity of the cellulase enzyme in *E. fetida* earthworms. This effect increased with increasing exposure time and concentration, and could be lethal. This might be one possible cause of the reduced body weight, since a reduction of cellulase activity compromises the feeding efficiency of exposed organisms, resulting in lower weight gain. The authors argued that this mechanism could also lead to acute toxicity in earthworms, which die following cellular autolysis caused by enzymatic inhibition. Such a phenomenon was observed in our study, in the form of leaking cellular liquids in dying worms following exposure to imidacloprid. Another possible explanation for the lethal effect involves a blocking of nervous system receptors by imidacloprid. While this effect of the neonicotinoid is more common in insects (Buffin, 2003), such blocking leads to an accumulation of acetylcholine, an important neurotransmitter, which results in muscle and organ paralysis and, depending on its intensity, can kill earthworms (Kidd and James, 1991).

The lack of lethality or body weight loss in earthworms exposed to fipronil and thiametoxam has been previously documented in studies of worm mortality with the same a.i. (NRA, 2001; Mostert et al., 2002). On the other hand, the lowest concentration of fipronil (Table 2) resulted in less weight loss than the control (Fig. 2), which could be the result of physiological stimuli caused by low concentrations of the insecticide. Some authors have reported sim-

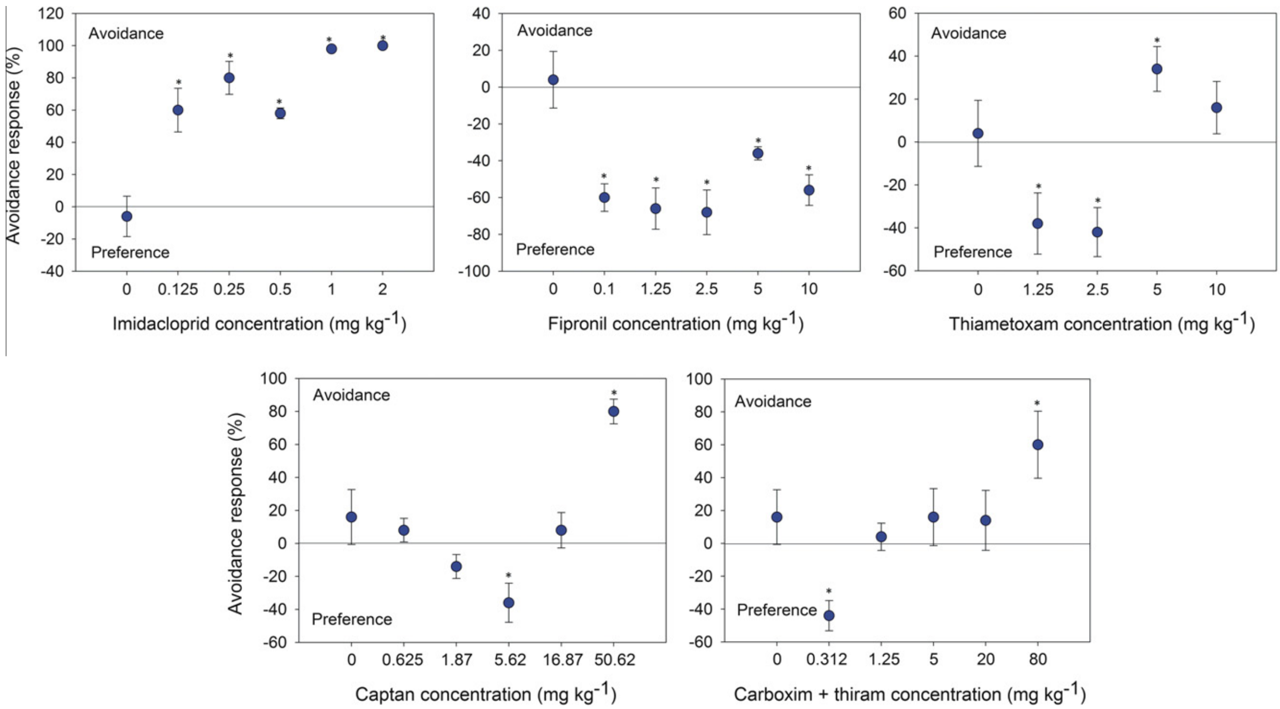

Fig. 3. Avoidance/preference responses of *E. andrei* in TAS treated with varying concentrations of the studied pesticides (mg kg^{-1} soil d.w.). *Means differ significantly from control (Fisher's exact test, $p < 0.05$).

ilar results and attributed the phenomenon to a hormetic response, i.e., the ability of a substance to be toxic at high concentrations but a stimulant at low concentrations (Zhang et al., 2009). It is worth emphasizing that while these insecticides did not kill earthworms in the acute toxicity tests, even at very high concentrations, this does not mean they have no lethal effects on the soil fauna; they are known to kill Isoptera (Peveling et al., 2003; Acda, 2007) and some non-target predatory soil arthropods (Kilpatrick et al., 2005; Moser and Obrycki, 2009).

As Anton et al. (1990) observed with captan, neither of the fungicides we studied caused mortality in *E. andrei*. For the fungicide Vitavax®, only the a.i. carboxin has shown evidence of acute toxicity to earthworms (EFSA, 2010). However, this fungicide is known to be lethal to aquatic organisms (EPA, 2004a, 2004b). There are other fungicides known to kill this earthworm species in soils. Römbke et al. (2007) observed mortality of *E. andrei* exposed to the fungicide Benomyl. Benomyl is considered highly toxic to *E. andrei* and in natural LUFA soil (ISO, 2003) showed an estimated LC_{50} of 66.8 mg kg^{-1} (soil d.w.), well above that observed in artificial soil (LC_{50} = 633 mg kg^{-1} soil d.w.). It may be the case that artificial substrates, while offering a good preliminary indicator tool, may partly hide the lethal effects of certain pesticides on earthworms. For that reason, confirmation that the fungicides we studied are not lethal to *E. andrei* awaits the results of tests in natural soils.

Our assessment of acute toxicity in earthworms revealed the lethal effects of pesticides on *E. andrei* in laboratory conditions. The toxicity tests are also important tools for choosing the sublethal concentrations (range-finder) for the more sensitive chronic toxicity and avoidance tests.

4.2. Chronic toxicity tests

As with the lethality tests, effects of chronic toxicity were strongest for imidacloprid, which showed a significant effect starting at concentrations of 0.75 mg kg^{-1} (soil d.w.) (LOEC). While the PEC (Table 1) was three times lower than this LOEC, it is worth noting that pesticide use is intensive and inadequate in many agricultural regions in Brazil, where the concentrations of pesticides introduced into soils may be higher than those estimated here and may put exposed organisms at risk (Nunes and Espíndola, 2012). In this case it is necessary to proceed to the next stage of pesticide risk assessment (semi-field or field tests), to validate the laboratory studies under more realistic conditions.

Gomez-Eyles et al. (2009) found fewer *E. fetida* juveniles (EC_{50} = 3.23 mg kg^{-1} soil d.w.) following exposure to imidacloprid, at levels similar to those in our study (EC_{50} = 4.07 mg kg^{-1} soil d.w.). These limitations in reproduction are probably linked to an increased number of anomalies in gamete formation, since Luo et al. (1999) have shown that starting at 0.2 mg kg^{-1} soil d.w., imidacloprid causes significant damage to spermatozoa of this species. This explanation was also supported by Capowiez and Berard (2006); Gomez-Eyles et al. (2009). Other possible explanations for the reduced reproductive rate are teratogenic effects or juvenile mortality. This seems unlikely, however, since Gomez-Eyles et al. (2009) observed a reduced production of cocoons, which supports the argument of Luo et al. (1999) regarding reduced fertility of adult worms.

With regard to the effect of thiametoxam on reduced reproduction in *E. andrei*, the NOEC we recorded (250 mg kg^{-1} soil d.w.) was much higher than that reported by EC (2007) (NOEC = 0.68 mg kg^{-1} soil d.w.) in their study of Actara® WG 25 (thiametoxam). A rigorous comparison of these contrasting results is not possible, however, because those authors did not describe the type of soil or other methodological details that could have influenced their results. Although this insecticide is a neonicotinoid, the same chemical class as imidacloprid, the EC_{50} value for thiametoxam was much higher than that calculated for imidacloprid (Table 3). According to Maienfisch et al. (2001), all insecticides of this class act on the nicotinic acetylcholine receptors, but each acts in a different fashion. Imidacloprid is currently being replaced by thiametoxam, which has the same effect but is considered less toxic to other non-target organisms (García-Chao et al., 2010).

Fipronil was the only pesticide we tested for which we could not calculate an EC_{50} value, since the number of juveniles was not reduced by >50% at the highest concentration tested (Table 2). While the LOEC and EC_{20} of this pesticide were lower than those observed for thiametoxam and captan (Table 3), the numbers suggest that among the pesticides tested fipronil was the least toxic to worm reproduction, since very high concentrations must be required to cause severe reductions in reproductive rate. In addition, the LOEC value for fipronil was much higher than the PEC (Table 1), suggesting low toxicity in the field. Studies similar to ours with fipronil formulations used to treat seeds also documented a reduced reproductive rate in the collembola *Folsomia candida* (San Miguel et al., 2008; Alves, 2010). Together with the studies of Daam et al. (2011), this information supports the idea that earthworms may not be ideal indicators of the risks posed to terrestrial fauna by insecticides and other similar substances (Jänsch et al., 2006).

Negative effects on the reproductive rates of soil fauna have been reported for captan (Ingham et al., 1991; Colinas et al., 1994) and carboxim + thiram (Toxiclin, 2001). This study provides new data on the chronic toxicity of these fungicides to earthworms (Table 3). Luo et al. (1999) argued that earthworms absorb most pesticides through the intestine, and RED (1999) indicate that most captan is metabolized in the gastrointestinal tract. Given that earthworms feed on soil organic matter and that the pesticides are absorbed by organic matter to varying degrees (Spark and Swift, 2002; Fenoll et al., 2011), it is possible that earthworms are exposed to captan not only through their skin but also during feeding, thereby increasing its toxicity to *E. andrei*. Reigart and Roberts (1999) argue that tetrahydrophthalimide, the main metabolite of captan, may be responsible for the harmful effects of the fungicide, by interacting inside cells with the sulfhydryl, hydroxyl, and amino enzyme groups, inhibiting metabolic processes (Waxman, 1998). While our study focused on worst-case scenarios, chronic toxicity of the fungicides was observed at 200 times PEC, indicating a low toxicity risk for earthworms in the field.

4.3. Avoidance tests

A relatively recent tool in terrestrial ecotoxicological screening, avoidance tests offer quick and inexpensive insights into risk analyses of pesticide pollution (Garcia et al., 2008; Daam et al., 2011; Cardoso and Alves, 2012). García-Santos and Keller-Forrer (2011) studied avoidance of *E. andrei* worms in soils treated with the pesticides carbofuran, chlorpyrifos, and metamidophos, and noted that if the same responses were repeated in the field, soil habitat quality could be compromised. Studies under tropical conditions have yielded similar results, thereby helping define acceptable concentrations of pesticides in soils (Nunes and Espíndola, 2012). Our study identified behavioral modifications for most of the studied pesticides at concentrations lower than those to which the other toxicity tests were sensitive (Table 3).

The LOEC for earthworm avoidance in soils treated with imidacloprid was the lowest concentration tested (Table 2). This concentration was almost two times lower than the PEC of this study, and almost three times lower than the PEC suggested by Oi (1999). Contrasting results were obtained by Capowiez and Berard (2006), who failed to observe an avoidance effect in the species *Aporrectodea nocturna* and *Allolobophora icterica* at an imidacloprid concentration of 1 mg kg^{-1} (soil d.w.) (Confidor® SL 200 g L^{-1}). They did, however, observe reduced body weight and digging activity. The authors attributed this to the fact that imidacloprid is toxic to but not an irritant for these species. It may be an irritant for *E. andrei*, given that worms avoided TAS treated with imidacloprid at concentrations lower than those that caused effects in the toxicity tests (Table 3).

In the case of thiametoxam, avoidance effects occurred at a concentration 100 times lower than the LOEC of the reproduction test (Table 3), suggesting that this pesticide may be more irritating than toxic for the worms. NRA (2001) also reported behavioral effects in oligochaetes exposed to this insecticide, including increased digging activity, but in our case effects were observed at a concentration of 1000 mg kg^{-1} (soil d.w.), much higher than the LOEC of our avoidance test.

In contrast to the avoidance observed for the other insecticides tested (Fig. 3), oligochaetes consistently preferred TAS treated with fipronil, regardless of concentration (Table 2). These results also are in conformity with to the observed exclusion of termites in agricultural areas treated with this insecticide (Rouland et al., 2003). There are two possible explanations for the behavior we observed: either fipronil is not an irritant for *E. andrei*, or, given that the highest tested concentration in the avoidance test was six times lower than the LOEC in the reproductive test (Table 3), the concentrations used were too low to produce an avoidance effect. In any case, the worms' preference for this pesticide, consistently observed across the range of tested concentrations, is intriguing (Fig. 3). Similar effects have been reported by Torkhani and Eržen (2011), who assessed the effect of ivermectin on *E. andrei* behavior. Those authors had no explanation for worms' preference for sites treated with the substance, but suggested that the most probable cause for the lack of avoidance was an incapacity of the chemoreceptors to detect ivermectin. Although earthworms are capable of distinguishing polluted soils, it is not known if their preference is determined by a lower biological availability of pollutants, by an incapacity of chemoreceptors to detect some substances, or other factors (Sousa et al., 2008).

Worms also preferred soils treated with low concentrations of both fungicides we tested, but in this case increased concentrations caused neither preference nor avoidance, and avoidance only occurred at the highest concentration tested for each pesticide (Fig. 3). This preference for low concentrations may also be interpreted as a hormetic response (Zhang et al., 2009), similar to that posited for fipronil and worm biomass. There are no reports of avoidance of captan and carboxim + thiram in earthworms. However, Garcia et al. (2008), working with artificial soils under tropical conditions, observed avoidance behavior in *E. fetida* exposed to the fungicides benomyl and carbendazim at concentrations of 3.2 mg kg^{-1} (soil d.w.). García-Santos and Keller-Forrer (2011) report significant variation in *E. fetida* behavior in the presence of mancozeb, and no avoidance at concentrations of 1000 mg kg^{-1} (soil d.w.). These differences in the effects of different concentrations of each pesticide is linked to different ways that fungicides act, as well as their interactions with soil attributes. It is worth noting that carboxim + thiram, despite having both contact and systemic effects (EPA, 2004a), caused less irritation in worms than captan. Worms avoided soils treated with captan concentrations four times lower than the LOEC of chronic toxicity, while the concentration of carboxim + thiram that had toxic effects on reproduction had no effect in avoidance tests (Table 3).

Various authors consider chronic toxicity tests to be the most sensitive among the first-tier tests of risk assessment (Amorim et al., 2005; Frampton et al., 2006; Novais et al., 2010). In this study we confirmed that chronic toxicity tests are more sensitive than acute toxicity tests. Given that only one tested pesticide caused mortality in *E. andrei*, while all tested pesticides reduced its reproductive rate (Table 3). However, most tested pesticides caused avoidance in the avoidance tests, at concentrations below the LOEC of the chronic toxicity tests.

5. Conclusions

All of the ecotoxicological tests we performed revealed negative effects on *E. andrei* for at least one of the pesticides tested. Only imidacloprid caused mortality in the worms, but all the pesticides had negative effects in the chronic toxicity and avoidance tests (except fipronil, which did not cause avoidance). The strength of these effects increased with increasing pesticide concentrations in the TAS. Imidacloprid was the most toxic of the tested substances, and caused impacts at lower concentrations than the other substances in all the assays. Avoidance behavior was detected at concentrations below the LOEC of the other tests for most pesticides (imidacloprid, thiametoxam, and captan), and avoidance tests were the most sensitive for these substances in the study. Reproduction tests were the most sensitive for fipronil and carboxim + thiram. More than one type of ecotoxicological assay should be employed in pesticide toxicological screening, since different criteria for assessment broaden the sensitivity of the risk assessment. These results offer new insights into the toxicity of pesticides used to treat seeds in tropical regions. Where high levels of toxicity were identified, we recommend the use of higher-tier tests to reduce the uncertainties in risk assessment.

Acknowledgements

P.R.L.A. thanks the Graduate Course in Agronomy of UEL (Universidade Estadual de Londrina) and the Soil Microbiology Laboratory of the Soil Science Department at ESALQ/USP (University of São Paulo) for the research opportunity, and the Agrisus Foundation for Sustainable Agriculture for the researcher grant (Project: PA-560/09). E.J.B.N.C. thanks National Counsel of Technological and Scientific Development (CNPq) for the researcher grant (No. 307563/2009-0). J.P.S. thanks CAPES for support in the cooperation project between University of Coimbra (Portugal) and ESALQ/USP (Brazil) (No. 198/08).

References

Acda, M.N., 2007. Toxicity of thiametoxam against Philippine subterranean termites. J. Insect Sci. 7, 1–6.

Alves, P.R.L., 2010. Avaliação Ecotoxicológica de Agrotóxicos em *Eisenia andrei* (Oligochaeta) e *Folsomia candida* (Collembola). Dissertação de Mestrado. Universidade Estadual de Londrina, Londrina.

Amorim, M.J.B., Römbke, J., Soares, A.M.V.M., 2005. Avoidance behaviour of *Enychytraeus albidus*: effects of benomyl, carbendazim, phenmedipham and different soil types. Chemosphere 59, 501–510.

Anton, F., Laborda, E., Laborda, P., 1990. Acute toxicity of the fungicide captan to the earthworm *Eisenia fetida* (Savigny). Bull. Environ. Contam. Toxicol. 45, 82–87.

Belfroid, A., Seinen, W., van Gestel, K., Hermens, J., 1993. The acute toxicity of chlorobenzenes for earthworms (*Eisenia andrei*) in different exposure systems. Chemosphere 26, 2265–2277.

Brühl, C.A., Guckenmus, B., Ebeling, M., Barfknecht, R., 2011. Exposure reduction of seed treatments through dehusking behaviour of the wood mouse (*Apodemus sylvaticus*). Environ. Sci. 18, 31–37.

Buffin, D., 2003. Imidacloprid. Pest. News 62, 22–23.

Capowiez, Y., Berard, A., 2006. Assessment of the effects of imidacloprid on the behavior of two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*) using 2D terraria. Ecotoxicol. Environ. Saf. 64, 198–206.

Cardoso, E.J.B.N., Alves, P.R.L., 2012. Soil Ecotoxicology. In: Begum, Ghousia (Ed.), Ecotoxicology. In Tech, Rijeka, pp. 27–50.

Colinas, C., Ingham, E., Molina, R., 1994. Population responses of target and nontarget forest soil organisms to selected biocides. Soil Biol. Biochem. 26, 41–47.

CONAMA, 2009. Critérios e valores orientadores de qualidade do solo quanto à presença de substâncias químicas e diretrizes para o gerenciamento ambiental de áreas contaminadas por essas substâncias em decorrência de atividades antrópicas. Resolução n. 420 de 28 de dezembro de 2009. Conselho Nacional do Meio Ambiente, Brasil.

Daam, M.A., Leitão, S., Cerejeira, M.J., Sousa, J.P., 2011. Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. Chemosphere 85, 1040–1047.

De Silva, P.M.C.S., Pathiratne, A., Van Gestel, C.A.M., 2010. Toxicity of chloropyrifos, carbofuran, mancozeb and their formulations to the earthworm *Perionyx excavatus* under tropical conditions. Appl. Soil Ecol. 44, 56–60.

EC, 2007. European Parliament and of the Council concerning the placing of biocidal products on the market. Regulation No. 1451/2007 of 4 December 2007 on the Second Phase of the 10-year Work Programme Referred to in Article 16(2) of Directive 98/8/EC of the O.J.L 325.

EC, 2009. European Parliament and of the council concerning the placing of plant protection products on the market and repealing: council directives 79/117/EEC and 91/414/EEC.Regulation No. 1107/2009 of The European Parliament and of the Council.

Edwards, C.A., 2004. Earthworm Ecology, 3. CRC Press LLC, Boca Raton.

EFSA, 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance carboxin. European Food Safety Authority Journal 8, 65pp.

EMBRAPA, 1999. Cuidados básicos para o plantio mecanizado de soja em Rondônia. Empresa Brasileira de Pesquisa Agropecuária, Brasil.

EPA, 2004a. Registration Eligibility Decision for Carboxin, List A Case 0012. EPA 738-R-04-015. United States Environmental Protection Agency, Washington.

EPA, 2004b. Registration Eligibility Decision for Thiram, List A Case 0122. EPA 738-R-04-012. United States Environmental Protection Agency, Washington.

FAO, 2012. FAOSTAT: Pesticides Trade – Import Value in Brazil. The statistics division, Food Agricultural Organization.

Fenoll, J., Ruiz, E., Flores, P., Hellín, P., Navarro, S., 2011. Reduction of the movement and persistence of pesticides in soil through common agronomic practices. J. Hazard. Mater. 85, 1375–1382.

Frampton, G.K., Jänsch, S., Scott-Fordsmand, J.J., Römbke, J., Van den Brink, P.J., 2006. Effects of pesticides on soil invertebrates in laboratory studies: a review and analysis using species sensitivity distributions. Environ. Toxicol. Chem. 25, 2480–2489.

Garcia, M., Römbke, J., De Brito, M.T., Scheffczyk, A., 2008. Effects of three pesticides on the avoidance behaviour of earthworms in laboratory test performed under temperate and tropical conditions. Environ. Pollut. 153, 450–456.

Garcia, M.V.B., 2004. Effects of Pesticides on Soil Fauna: Development Of Ecotoxicological Test Methods for Tropical Regions. Ecology and Development Series, vol. 19. University of Bonn, Germany.

García-Chao, M., Agruña, M.J., Calvetea, G.F., Sakkas, V., Llompart, M., Dagnac, T., 2010. Validation of an off line solid phase extraction liquid chromatography–tandem mass spectrometry method for the determination of systemic insecticide residues in honey and pollen samples collected in apiaries from NW Spain. Anal. Chim. Acta 672, 107–113.

García-santos, G., Keller-forrer, K., 2011. Avoidance behaviour of *Eisenia fetida* to carbofuran, chlorpyrifos, mancozeb and metamidophos in natural soils from the highlands of Colombia. Chemosphere 84, 651–656.

Gomez-Eyles, J.L., Svendsen, C., Lister, L., Martin, H., Hodson, M.E., Spurgeon, D.J., 2009. Measuring and modeling mixture toxicity of imidacloprid and thiacloprid on *Caenorhabditis elegans* and *Eisenia fetida*. Ecotoxicol. Environ. Saf. 72, 71–79.

Ingham, E.R., Parmelee, R., Coleman, D.C., Crossley, D.A., 1991. Reduction of microbial and faunal groups following application of streptomycin and captan in Georgia no-tillage agroecosystems. Pedobiologia 35, 297–304.

ISO, 1998. Soil Quality: Effects of Pollutants on Earthworms (Eisenia fetida) – Part 2: Method for the Determination of Effects on Reproduction. No. 11268-2. International Organization for Standardization. Geneva, Switzerland.

ISO, 2003. Soil Quality: Guidance on the Ecotoxicological Characterization of Soils and Soil Materials. No. 15799:2003. International Organization for Standardization. Geneva, Switzerland.

ISO, 2008. Soil Quality: Avoidance Test for Testing the Quality of Soils and Effects of Chemicals on Behavior – Part 1: Test with Earthworms (Eisenia fetida and Eisenia andrei). ISO/DIS 17512-1.2. International Organization for Standardization. Geneva, Switzerland.

Jänsch, S., Frampton, G.F., Römbke, J., Van den Brink, P.J., Scott-Fordsmand, J.J., 2006. Effects of pesticides on soil invertebrates in model ecosystem and field studies: a review and comparison with laboratory toxicity data. Environ. Toxicol. Chem. 25, 2490–2501.

Kidd, H., James, D.R., 1991. The Agrochemicals Handbook, Third ed. Royal Society of Chemistry Information Services, Cambridge.

Kilpatrick, A.L., Hagerty, A.M., Turnipseed, S.G., Sullivan, M.J., Bridges, W.C., 2005. Activity of selected neonicotinoids and dicrotophos on nontarget arthropods in cotton: implications in insect management. J. Econ. Entomol. 98, 814–820.

Kreutzweiser, D.P., Good, K.P., Chartrand, D.T., Scarr, T.A., Holmes, S.B., Thompson, D.G., 2008. Effects on litter-dwelling earthworms and microbial decomposition of soil-applied imidacloprid for control of wood-boring insects. Pest Manage. Sci. 64, 112–118.

Kula, H., Larink, O., 1997. Development and standardization of test methods for the prediction of sublethal effects of chemicals on earthworms. Soil Biol. Biochem. 29, 635–639.

Luo, Y., Zang, Y., Zhong, Y., Kong, Z., 1999. Toxicological study of two novel pesticides on earthworm *Eisenia fetida*. Chemosphere 39, 2347–2356.

Maienfisch, P., Angst, M., Brancl, F., Fischer, W., Hofer, D., Kayser, H., Kobel, W., Rinclisbacher, A., Senn, R., Steinemann, A., Widmer, H., 2001. Chemistry and biology of thiametoxam: a second generation neonicotinoid. Pest Manage. Sci. 57, 906–913.

Moser, S.E., Obrycki, J.J., 2009. Non-target effects of neonicotinoid seed treatments; mortality of Coccinellid larvae related to zoophytophagy. Biol. Control 51, 487–492.

Mostert, M.A., Schoeman, A.S., Van Der Merwe, M., 2002. The relative toxicities of insecticides to earthworms of the Pheretima group (Oligochaeta). Pest Manage. Sci. 58, 446–450.

Munkvold, G.P., Sweets, L., Wintersteen, W., 2006. Iowa Commercial Pesticide Applicator Manual. Seed Treatment. Iowa State University, Ames.

Natal-da-Luz, T., Römbke, J., Sousa, J.P., 2008. Avoidance tests in site-specific risk assessment: influence of soil properties on the avoidance response of collembola and earthworms. Environ. Toxicol. Chem. 27, 1112–1117.

Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2010. Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing? Chemosphere 79, 233–237.

NRA, 2001. Evaluation of the new active thiametoxam in the product Cruiser 350 FS insecticide seed treatment. National registration authority for agricultural and veterinary chemicals, Canberra.

Nunes, M.E.T., Espíndola, E.L.G., 2012. Sensitivity of *Eisenia andrei* (Annelida, Oligochaeta) to a commercial formulation of abamectin in avoidance tests with artificial substrate and natural soil under tropical conditions. Ecotoxicology 21, 1063–1071.

OECD, 1984. Guideline for Testing of Chemicals No. 207: Earthworm Acute Toxicity Test. Organization for Economic Co-operation and Development, Paris.

OECD, 2004. Guideline for Testing of Chemicals No. 222, Earthworm Reproduction Test (Eisenia fetida/andrei). Organization for Economic Co-operation and Development. Paris, France.

Oi, M., 1999. Time-dependent sorption of imidacloprid in two different soils. J. Agric. Food Chem. 47, 327–332.

Pesaro, M., Widmer, F., Nicollier, G., Zeyer, J., 2003. Effects of freeze-thaw stress during soil storage on microbial communities and methidathion degradation. Soil Biol. Biochem. 35, 1049–1061.

Peveling, R., William, A.N.M., Nagel, P., Rasolomanana, H., Raholijaona, Rakotomianina, L., Ravoninjatovo, A., Dewhurst, C.F., Gibson, G., Rafanomezana, S., Tingle, C.C.D., 2003. Impact of locust control on harvester termites and endemic vertebrate predators in Madagascar. J. Appl. Ecol. 40, 729–741.

RED, 1999. Captan: EPA-738-R-99-015; US Environmental Protection Agency, Office of Pesticide Programs. Registration Eligibility Decision, Washington.

Reigart, J.R., Roberts, J.R. Organophosphate Insecticide. Recognition and Management of Pesticide Poisonings, In: 5th ed., EPA 735-R-98-003; U.S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances, US Government Printing Office, Washington.

Römbke, J., Garcia, M.V.B., Scheffczyk, A., 2007. Effects of the fungicide benomyl on earthworms in laboratory tests under tropical and temperate conditions. Arch. Environ. Contam. Toxicol. 53, 590–598.

Rouland, C., Lepage, M., Chotte, J.L., Diouf, M., Ndiaye, D., Ndiaye, S., Seugé, C., Brauman, A., 2003. Experimental manipulation of termites (Isoptera, Macrotermitinae) foraging patterns in a Sahelo-Sudanese savanna: effect of litter quality. Insect. Soc. 50, 309–316.

San Miguel, A., Raveton, M., Lempérière, G., Ravanel, P., 2008. Phenylpyrazoles impact on *Folsomia candida* (Collembola). Soil Biol. Biochem. 40, 2351–2357.

Sousa, A., Pereira, R., Antunes, S.C., Cachada, A., Pereira, E., Duarte, A.C., Gonçalves, F., 2008. Validation of assays for the screening assessment of soils under different anthropogenic disturbances. Ecotoxicol. Environ. Safe. 71, 661–670.

Spark, K.M., Swift, R.S., 2002. Effect of soil composition and dissolved organic matter on pesticide sorption. Sci. Total Environ. 298, 147–156.

Torkhani, A.L., Eržen, N.K., 2011. Does ivermectin attract earthworms? J. Soils Sedim. 11, 124–128.

Toxiclin, 2001. Ficha de Informações de Segurança de Produto Químico – Vitavax® Thiram 200 SC. FISPQ – AG 05/02, Brasil.

Waxman, M.F., 1998. Agrochemical and Pesticides Safety Handbook. Lewis Publishers, Boca Raton.

Zhang, Y., Shen, G., Yu, Y., Zhu, H., 2009. The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*. Environ. Pollut. 157, 3064–3068.