Ecotoxicology and Environmental Safety 105 (2014) 65–71

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Seed dressing pesticides on springtails in two ecotoxicological laboratory tests

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

[a] Department of Soil Science, University of São Paulo, Avenida Pádua Dias, 10, 13416-900 Piracicaba, Brazil
[b] IMAR-CMA, Department of Life Sciences, University of Coimbra, P3004-517 Coimbra, Portugal
[c] Department of Agronomy, State University of Londrina, 86051990 Londrina, Brazil

ARTICLE INFO

Article history:
Received 27 January 2014
Received in revised form
3 April 2014
Accepted 9 April 2014

Keywords:
Seed treatment
Collembola
Folsomia candida
Environmental risk assessment
Soil fauna
Soil ecotoxicology

ABSTRACT

Terrestrial ecotoxicological tests are powerful tools for assessing the ecological risks that pesticides pose to soil invertebrates, but they are rarely used to evaluate seed dressing pesticides. This study investigated the effects of seed dressing pesticides on survival and reproduction of *Folsomia candida* (Collembola), using standardized ecotoxicological tests (after ISO guidelines with few adaptations for tropical conditions). Commercial formulations of five seed dressing pesticides were tested individually in Tropical Artificial Soil (TAS): the insecticides imidacloprid, fipronil, thiametoxam, and the fungicides captan and carboxin + thiram. Thiametoxam, captan, and carboxin + thiram were only lethal to *F. candida* at the highest concentration tested (1000 mg of active ingredient kg^{-1} of dry soil). Imidacloprid and fipronil were lethal at lower concentrations (100 and 10 mg a.i. kg^{-1} soil d.w, respectively), however, these concentrations were much higher than those predicted (PEC) for soil. Imidacloprid and fipronil were the most toxic pesticides in both tests, reducing significantly collembolan reproduction (EC_{20} = 0.02 and 0.12 mg a.i. kg^{-1} soil d.w, respectively). Further studies under more realistic conditions are needed, since imidacloprid and fipronil reduced collembolan reproduction at concentrations below or close to their respective PECs.

1. Introduction

Treating seeds with pesticides is an important and increasingly common practice in agriculture. According to FAO (2012), in the last twenty years we have seen a significant increase worldwide in the use of seed dressing pesticides. The method is considered one of the most efficient available for preventing or minimizing damage by pests and pathogens that attack seeds and seedlings early in the crop cycle (Munkvold et al., 2006). Because the method is simple to apply and has a low cost-benefit ratio, the Brazilian market for seed dressing fungicides has more than doubled in size over the last decade (Menten and Moraes, 2010), and there is evidence that nearly 100 percent of soybean seeds are now treated with fungicides and 30 percent with insecticides (Baudet and Peske, 2006).

Although treating seeds with pesticides contributes indirectly to higher crop productivity, it also generates residues that can be toxic to non-target organisms. These residues can cause poisoning in mammals, phytotoxicity in plants, impacts on aquatic and soil communities, and leave pesticide traces in food products (Paulsrud et al., 2001). In this context, increased amounts of pesticide residues in agricultural soils imply a greater threat to soil fauna. In turn, impacts on the soil fauna may impair the processes they drive, including those related to organic matter decomposition, nutrient cycling, and maintenance of soil structure (Lavelle et al., 2006).

In the European Union, the assessment of pesticide impacts on soils is regulated by specific guidelines for approving the sale of plant-protection products (EC, 2013). These guidelines include standardized methods to assess the toxic effects of pesticides on living non-target organisms. In terrestrial environments, three invertebrate species are mostly recommended for ecotoxicological assays: *Eisenia fetida/andrei* (Lumbricidae), *Folsomia candida* (Collembola), and *Enchytraeus albidus/crypticus* (Enchytraeidae) (Jänsch et al., 2006). In line with these international guidelines, Brazil has developed specific laws to manage polluted sites, and Brazilian legislation now includes several criteria for assessing soil quality via the use of live organisms. These include the establishment of ecological risk guidance values for pesticides in soil,

* Corresponding at: Department of Soil Science Avenida Pádua Dias, No. 11, 13416-900 Piracicaba, São Paulo, Brazil. Fax: +55 19 3417 2110.
E-mail address: pauloroger@usp.br (P.R.L. Alves).

http://dx.doi.org/10.1016/j.ecoenv.2014.04.010
0147-6513/

which are obtained via traditional methods of terrestrial ecotoxicology (CONAMA, 2009).

While springtails account for a small proportion of soil biomass and respiration (Coleman et al., 2004; Jänsch et al., 2005), these arthropods have a significant influence on microbial ecology and soil fertility, via their role in regulating processes of decomposition and nutrient cycling (Culik and Zeppelini, 2003). This is one reason why several collembolan species have been used as bioindicators of pesticide toxicity in soils (Achazi et al., 2000; Greenslade and Vaughan, 2003; Heupel, 2002). *F. candida* is the collembolan species most commonly used in standard ecotoxicological tests (ISO, 1999). This species has a short generation time and reproduces by parthenogenesis, which makes it especially appropriate for tests that require analyses both at individual and population level in a single assay (Jänsch et al., 2005). In addition, Frampton et al. (2006) have argued that in laboratory toxicity studies *F. candida* is more sensitive to a broad range of pesticide modes of action (including biocide, fungicide, herbicide, and insecticide) than the earthworm *E. fetida*. More recently, Daam et al. (2011), when evaluating the sensitivity of pesticides in several groups of soil invertebrates when compared to the sensitivity of *Eisenia* species, found that collembolans are much more sensitive towards insecticides. Although earthworms (soft bodied organisms with major uptake routes being via the direct contact with soil solution through the skin and via ingestion of soil particles) may be more susceptible to the uptake of pesticides than collembolans (hard-bodied organisms with an exoskeleton and uptaking water via specialized organs) (Peijnenburg et al., 2012), the toxicity of these substances is driven not only by exposure but mainly by their mode of action. Most insecticides have relatively higher accessibility to insect's nervous system (or other metabolic pathways, depending on the insecticide class), so are expected to cause higher toxicity on collembolans than on earthworms (Marrs and Ballantyne, 2004). For these reasons, the use of collembolans in ecotoxicological laboratory tests has become increasingly common over the last decade (Heupel, 2002; Liu et al., 2012; Natal-da-Luz et al., 2009; Santos et al., 2010) and is now mandatory in the new data requirements for pesticide risk assessment when these products are applied directly to the soil (EC, 2013). To date, the ecotoxicological studies that have investigated standard invertebrate species under tropical conditions have used earthworms (De Silva and Van Gestel, 2009; De Silva et al., 2010; Garcia et al., 2008; Nunes and Espíndola, 2012) and only very few used collembolans (Chelinho et al., 2012; Niemeyer et al., 2010). Likewise, the ecological risks posed by seed dressing pesticides have not been widely studied in tropical regions (Alves et al., 2013).

Among the various formulations of pesticides used to treat seeds, the insecticides Gaucho®, Cruiser® and Standak®, with the active ingredients (a.i.) imidacloprid, thiametoxam and fipronil, respectively, and the fungicides Captan® (a.i. captan), and Vitavax® (a.i. carboxin+ +thiram), are widely used in agriculture and their effects on earthworms were already studied in laboratory ecotoxicological assays by Alves et al. (2013). According to the literature, these active ingredients can also be toxic to collembolans (EFSA, 2010; Heijbroek and Huijbregts, 1995; Peck, 2009; Rather and Shah, 2010; Reynolds, 2008; San Miguel et al., 2008) and other non-target soil organisms like predatory mites, coleopteran larvae and spiders (Jackson and Ford, 1973; Moser and Obrycki, 2009; Tingle et al., 2000). In order to increase the understanding of the ecotoxicological effects of seed dressing pesticides on soil fauna, survival and reproduction tests with *F. candida* (Collembola) were performed with soils contaminated with five seed dressing pesticides. The usefulness of either survival or reproduction tests for the evaluation of the potential environmental risks of these products was also discussed.

2. Material and Methods

2.1. Test organisms and test conditions

A laboratory culture of *F. candida* (Collembola) of European origin was established, following methods adapted from ISO standard 11268-2 (ISO, 1999). The organisms were cultured in cylindrical plastic vials containing a mixture of activated charcoal (dust), water, and plaster of Paris in the proportion 1:7:11 (w:w:w). Granulated dry yeast (*Saccharomyces cerevisiae*) was supplied weekly as food.

F. candida of ten to twelve days old, taken from synchronized cultures, were used in the ecotoxicological assays. The laboratory cultures and all bioassays were carried out in a climate-controlled room with a temperature of 23 ± 2 °C and a 12:12 h light/dark photoperiod [slightly modified ISO (1999)], trying to mirror tropical conditions.

2.2. Artificial soil and tested contaminants

The ecotoxicological assays were carried out with Tropical Artificial Soil (TAS), an adaptation of OECD artificial soil (OECD, 1984) for tropical conditions, also used by De Silva and Van Gestel (2009) and Rombke et al. (2007). The TAS consisted of fine sand (> 50 percent of particles measuring between 0.05 and 0.2 mm), kaolinitic clay (powdered kaolinite) and powdered coconut husk in the proportion of 7:2:1 (w:w:w). The pH of TAS was corrected to 6.0 ± 0.5 with the addition of calcium carbonate ($CaCO_3$). The water-holding capacity (WHC) of TAS was determined following ISO (1999). Before the beginning of the tests, soil moisture was corrected to a mean value of 60 percent of the WHC, using water for the control and diluted pesticide solutions for the treatments. At the start (after the application of pesticide solutions/suspensions) and at the end of each bioassay, the soil pH was measured (1 M KCl, 1:5, w/v) following ISO (1999).

The formulations of the insecticides imidacloprid, thiametoxam and fipronil, and of the fungicides captan and carboxin+thiram, were chosen based on their widespread use to treat seeds in Brazilian crops:

Gaucho® (Bayer AG), a neonicotinoid insecticide with the active ingredient (a.i.) imidacloprid (IUPAC: (E)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine), is active against sucking insects because of its unique plant-systemic and translaminar properties (Marrs and Ballantyne, 2004). It causes irreversible blockage of the acetylcholine receptors of insect's nervous system, which leads to an accumulation of this neurotransmitter, resulting in paralysis and sometimes death (Kidd and James, 1991).

Cruiser® (Syngenta) is the second-generation neonicotinoid insecticide, belonging to the sub-class of thianicotinyl, with the a.i. thiametoxam (IUPAC: (EZ)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro)amine) (Maienfisch et al., 2001). It has a broad-spectrum insecticidal mode of action, focused on the nicotinic acetylcholine receptors of insects. The molecule mimics the chemical messenger acetylcholine and binds to its receptor site, causing irreversible harm to the nervous system that at high intensities can cause death in invertebrates (NRA, 2001).

The insecticide Standak® (BASF) is a phenylpyrazole insecticide with the a.i. fipronil (IUPAC: 5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile). It has a broad-spectrum activity and acts directly on the central nervous system of organisms, where it inhibits the gamma aminobutyric acid (GABA) receptor, a neurotransmitter responsible for regulating neuronal excitability and preventing excessive nerve stimulation. This inhibition causes death in sensitive individuals (Coutinho et al., 2005).

Captan® (Milenia Agrosciences) is a non-systemic thiodicarboximide fungicide which has protective and curative action, with the a.i. captan (IUPAC: *N*-(trichloromethylthio)cyclohex-4-ene-1,2-dicarboximide). Its mode of action is linked to an intracellular interaction with the sulfhydryl, hydroxyl, and amino enzyme groups, leading to an inhibition of some metabolic processes (Waxman, 1998).

Vitavax® (Chemtura) is a mixture of the a.i. carboxin (IUPAC: 5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide)+thiram (IUPAC: tetramethylthiuram disulfide), which are in the oxathiin and dithiocarbamate fungicide classes, respectively. Carboxin is systemic and inhibits the dehydrogenation of succinic acid to fumaric acid, an important step in the tricarboxylic acid cycle (Stenersen, 2004). Thiram has contact action and inhibits the alcohol dehydrogenase enzymes, which can lead to toxicity by co-exposure to ethanol (Marrs and Ballantyne, 2004).

The predicted environmental concentrations (PEC) of these tested pesticides (Table 1) were estimated based on the calculation of the volume of each pesticide required to treat enough seeds to plant one hectare (ha) with soybeans, according to Alves et al. (2013). The commercial formulations used were diluted in deionized water and the pesticide solutions (test treatments with pesticide contamination based on active ingredients), or deionized water (control), were applied to the TAS, such that the solutions/suspensions were distributed evenly throughout the soil. Increasing concentrations of the a.i. (0; 1.0; 10; 100; 500; 1000 mg a.i. kg^{-1} of soil dry weight, DW) were used in the acute toxicity tests. For chronic toxicity assays, the increasing sub-lethal concentrations used (0; 0.06; 0.12; 0.25; 0.50; 1.0 mg a.i. kg^{-1} soil DW) were based on the results obtained in the acute toxicity tests and on PEC values (Table 1).

2.3. Acute toxicity test

The lethal potential of the pesticides to *F. candida* was assessed via an acute ecotoxicity test (ISO, 1999). Cylindrical glass containers (35 × 115 mm, diameter/height) were filled with 30 g of artificial soil (fresh weight – FW) containing the pesticide concentrations (treatments) or deionized water (control). Six replicates per pesticide concentration were prepared (five of them containing springtails and one extra for pH and moisture determination at the end of the assay). At the start of the test, ten collembolans were introduced in each experimental unit that was immediately closed with a hermetic seal. At the start of the tests about 2 mg of granulated dry yeast was added as food in each container. After seven days the containers were opened to allow gas exchange. After fourteen days of exposure, the content of each container was carefully transferred to a larger container, which was filled with water, such that the surviving individuals floated on the water surface. Drops of black ink were added to the water to increase the visual contrast between collembolans and the liquid. Collembolans were counted visually to calculate the average percentage survival in each treatment.

2.4. Chronic toxicity test

The effects of the pesticides on *F. candida* reproduction were assessed using the chronic toxicity tests, based on the methods described in the ISO guideline 11267 (ISO, 1999). Six replicates per pesticide concentration were prepared, as explained above. The procedures were all identical to those explained in acute toxicity test, except for the duration, that was of 28 days, after when the collembolans were visualized by flotation, as in the mortality test. In this case, however, the number of juveniles was determined by photographing the water surface for each replicate. The images were analyzed with UTHSCSA Image Tool 3.0 software to quantify the juveniles in each treatment.

2.5. Data analysis

Results of the acute toxicity tests were expressed as the average percentage of surviving organisms, while the average number of juveniles was counted in the chronic test. Significant differences between treatments were tested through analysis of variance (ANOVA, $p \leq 0.05$) in both acute and chronic toxicity tests. Treatments were compared with the control through post-hoc Dunnett's test, using the R software package, version 2.5.1. These differences were used to establish NOEC (No observed effect concentration) and LOEC (Lowest observed effect concentration) values for the toxicity tests.

In addition, PriProbit® 1.63 software was used to estimate the LC_{50} (lethal concentration of 50 percent) values in the acute toxicity tests. For the chronic toxicity tests, non-linear regressions using logistic, exponential, or hormetic models were performed to estimate the EC_{20} and the EC_{50} values (the concentration that reduces collembolan reproduction by 20 and 50 percent, respectively) using Statistica® 7.0 software.

3. Results

3.1. Test validation

F. candida mortality was < twenty percent in the controls of the acute and chronic toxicity tests, fulfilling the validity criteria required in ISO (1999). In chronic toxicity assays, the number of juveniles was ≥ 100 individuals in all replicates, and the coefficient of variation (CV) was < 30 percent for the control.

3.2. Acute toxicity responses

All the pesticides tested were lethal to *F. candida*, but the toxicity level obtained was dependent on the a.i. (Table 2). Only the insecticides imidacloprid and fipronil yielded mortality levels that were sufficient to estimate the 50 percent lethal concentration (LC_{50}=20.96 and 59.62 mg a.i. kg^{-1} DW, respectively) for collembolans. Therefore, these pesticides were the most lethal ones for *F. candida*. The insecticide thiametoxam and the fungicides captan and carboxin+thiram were only lethal at the highest concentration tested (1000 mg a.i. kg^{-1} DW).

3.3. Chronic toxicity responses

Concentrations of captan, carboxin+thiram and thiametoxam in TAS had no significant effect on the reproductive performance of *F. candida,* while the insecticides imidacloprid and fipronil caused a significant reduction in the number of juveniles produced by the collembolans in concentrations from 0.06 to 0.12 mg a.i. kg^{-1} DW, respectively (the LOEC values; Fig. 1). In spite of the toxicity caused by these insecticides, in the highest tested concentration the reduction in reproduction was always < 50 percent of that observed in the

Table 1
Commercial names, manufacturers and active ingredients (a.i.) of the pesticides studied, and their predicted environmental concentrations (PEC) at the commercially recommended doses for soybean crops (Adapted from Alves et al., 2013).

Commercial name	manufacturer	a.i. name	a.i. content (g L^{-1})	PEC (mg of a.i. kg^{-1} dry soil)
Gaucho® 600 FS	Bayer AG	imidacloprid	600	0.230
Standak® 250 SC	BASF	fipronil	250	0.096
Cruiser® 350 FS	Syngenta	thiametoxam	350	0.201
Captan® 480 SC	Milenia Agrosciences	captan	480	0.230
Vitavax® 200 SC	Chemtura	carboxin+thiram	200	0.115

Table 2
Toxic values estimated based on pesticide effects on *F. candida* in acute (LC_{50}) and chronic toxicity tests (LOEC, NOEC and EC_{20}).

Test	Parameter	Active ingredient (mg kg^{-1} soil DW)				
		imidacloprid	fipronil	thiametoxam	captan	carboxin+thiram
Acute	**NOEC**	10	1	500	500	500
	LOEC	100	10	1000	1000	1000
	Upper limits (95 percent)	32.13	88.25	n.d.	n.d.	n.d.
	LC_{50}	20.96	59.62	> 1000	> 1000	> 1000
	Lower limits (95 percent)	9.51	30.99	n.d.	n.d.	n.d.
Chronic	**NOEC**	< 0.06	0.06	1	1	1
	LOEC	0.06	0.12	> 1	> 1	> 1
	Upper limits (95 percent)	0.021	0.197	n.d.	n.d.	1.227
	EC_{20}	0.010	0.120	> 1	> 1	0.373
	Lower limits (95 percent)	0.001	0.044	n.d.	n.d.	0.048

n.d. – data do not allow estimation of a 95 percent confidence interval.

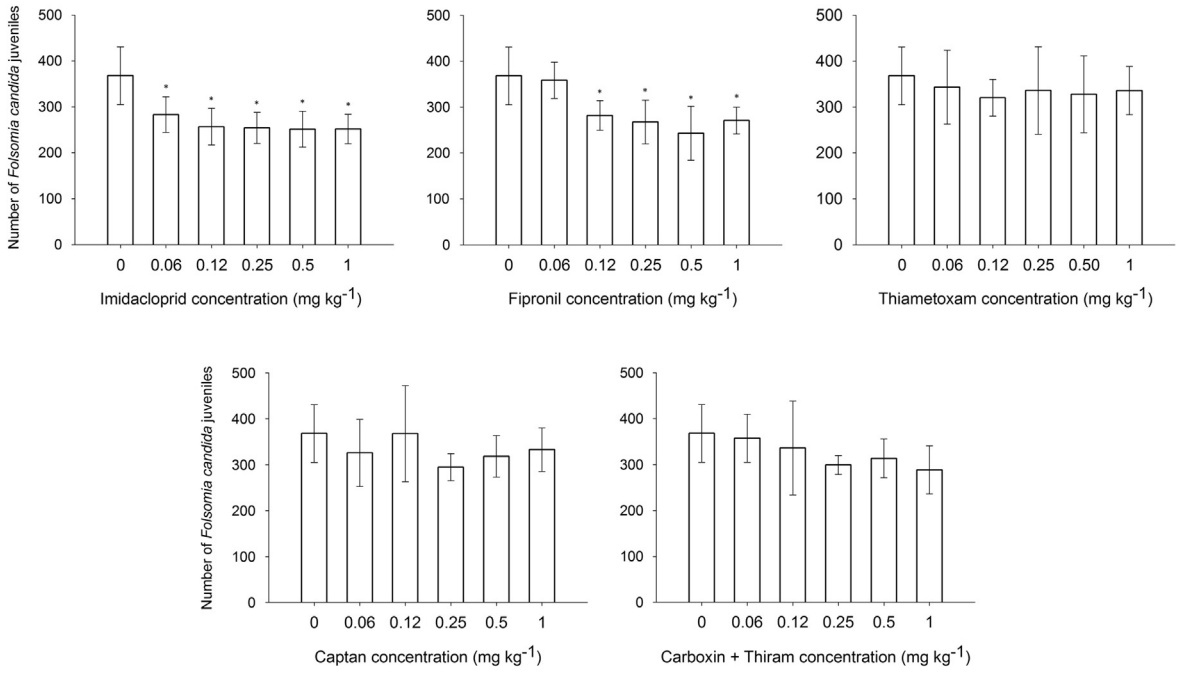

Fig. 1. Mean number of *Folsomia candida* juveniles (± standard deviation, $n=5$) in chronic toxicity tests using Tropical Artificial Soil (TAS) treated with increasing concentrations of the seed dressing pesticides, after 28 days. Bars with asterisks (*) indicate significant difference in reproduction levels between treatment and control.

control, which did not allow estimation of the EC_{50} values and therefore we estimate the EC_{20} values (Table 2).

4. Discussion

4.1. Fipronil

While fipronil was the most lethal pesticide tested for *F. candida* (Table 2), the NOEC value (1 mg kg^{-1} soil DW) of this insecticide in the acute toxicity test is ten times higher than its PECsoil (0.096 mg a.i. kg^{-1} DW; Table 1). Similar results have been reported by San Miguel et al. (2008), who obtained a NOEC of 0.5 mg kg^{-1} soil DW for *F. candida*, almost five times the PEC we estimated (Table 1). However, there may be other factors that influence fipronil's lethality to *F. candida*, such as the stage of development of the collembolans. San Miguel et al. (2008) argued that toxicity may be stronger in juveniles than in adults. While our results suggest a low lethal potential of fipronil for adult collembolans, we did not set up an acute toxicity test with juveniles in order to quantify these effects on the various developmental stages present in the soil. Fipronil is also known to be lethal (LOEC = 3.2 g ha^{-1} of a.i.) for *Coarctotermes clepsydra* (Isoptera: Termitidae) (Peveling et al., 2003), as well as for other non-target soil organisms, including beetles, predatory mites, and spiders (as reviewed by Tingle et al., 2000).

The chronic toxicity tests indicate that fipronil used for seed dressing was toxic to *F. candida* (Fig. 1). This pesticide caused a decline in reproduction in *F. candida* with the EC_{20} = 0.12 mg kg^{-1} soil DW. Similar results were reported by San Miguel et al. (2008), who found significant declines of 50 percent of the number of juveniles of *F. candida* (EC_{50}) at fipronil concentrations between 0.335 and 0.50 mg kg^{-1} soil DW. Although both studies found effects at concentrations that are slightly higher than fipronil's PEC in soil, this is not sufficient to dismiss the risk to collembolans in the field, since in many Brazilian agricultural areas pesticides are applied at much higher doses than the PEC values we estimated (Nunes and Espíndola, 2012). It is also important to note that the organic matter (OM) fraction of soils can influence toxicity, because of its pesticide sorption capacity, leading to different toxic values of the effective concentration of a given pesticide. Martikainen and Krogh (1999), studying the insecticide dimethoate, reported that the EC_{50} for collembolans was higher in OECD Artificial Soil with 10 percent OM (8.6 percent humus) than in samples of the same soil with 5 percent and 1 percent OM (4.8 and 1.8 percent humus, respectively). These authors also found that the EC_{50} of the same insecticide was higher in artificial soil than in natural soils with the same amount of humus. If indeed the toxicity of the insecticide fipronil is influenced by the OM of the artificial soil, as is the case with dimethoate (Martikainen and Krogh, 1999), then the EC_{20} value obtained in our study (TAS with 10 percent OM) may underestimate the true toxicity of the product for collembolans in soils of tropical regions, where OM soil content is typically lower (Six et al., 2002). In similar situations, the use of natural soils in the laboratory tests could help to identify if the OM factor is responsible for the toxicity reduction of the pesticide. However, if the uncertainty on ecological risk still remains, Jänsch et al., 2006 argue that it is crucial to proceed to the next stage of pesticide risk assessment, in semi-field or field conditions, in order to corroborate the results obtained in laboratory conditions.

4.2. Imidacloprid

Although imidacloprid showed a higher NOEC (10 mg kg^{-1} soil DW) than fipronil (1 mg kg^{-1} soil DW) in the acute toxicity assays, the LC_{50} of this neonicotinoid was lower than for fipronil (Table 2), indicating that, even if fipronil starts killing at lower concentrations, imidacloprid has a higher potential to cause lethality in *F. candida*. Reynolds (2008) found an LC_{50} for *F. candida* of 1.38 mg a.i. kg^{-1} DW, that is much lower than that estimated in our study (LC_{50} = 20.96 mg a.i. kg^{-1} DW). According to Diogo et al. (2007) the sensitivity of *F. candida* towards contaminants (e.g. pesticides) is dependent on the strain of the individuals. In any case, the PEC of imidacloprid (Table 1) was below the estimated LC_{50} value. It has been reported that imidacloprid kills other non-target soil invertebrates, including earthworms with LC_{50} of 25.53 mg a.i. kg^{-1} DW

(Alves et al., 2013) and predatory coleopterans with LC_{50} of 11.54 ppm of the a.i. (He et al., 2012). The different LC_{50} values found for imidacloprid for different components of the soil fauna have been attributed in part to different test methods and/or conditions, but are primarily associated with the selective toxicity of the neonicotinoid for different species/strains (Tomizawa and Casida, 2005).

Imidacloprid was the most toxic pesticide in chronic toxicity tests (Table 2). The EC_{20} obtained for this insecticide (0.01 mg kg^{-1} DW) was more than twenty times lower than the soil concentration expected after using treated seeds (PEC=0.23 mg a.i. kg^{-1} DW). This suggests that springtail populations in fields where seeds treated with this compound may be at risk, as well as the biological processes in which they play a role (Rusek, 1998). These considerations would corroborate the results of Peck (2009), who reports significant suppression on the abundance of Entomobryomorpha (same order as *F. candida*) over three years, when using 0.37 kg of imidacloprid ha^{-1} (dose in the recommended range of application of Merit 0.5G) in field plots. Kreutzweiser et al. (2009) also reported that sub-lethal doses of imidacloprid can cause adverse effects on non-target decomposer invertebrates, what is prone to affect the decomposition process of leaf litter.

4.3. Thiametoxam

Thiametoxam caused mortality to *F. candida* only at 1000 mg a.i. kg^{-1} DW. The low toxicity found in the acute test agrees with the outcome of the chronic reproduction test, in which no statistical differences were found between collebolans' reproduction in treatments and control (Table 1).

This concentration also greatly exceeds the PEC of this pesticide (0.201 mg a.i. kg^{-1} DW; Table 1). In spite of the low toxicity of thiametoxam found for *F. candida* in acute tests, this insecticide is known to be lethal for isopterans at 0.41–0.64 ppm of a.i. (Acda, 2007) and for larvae of the predaceous coccinellid at 0.250 mg a.i. $seed^{-1}$ (Moser and Obrycki, 2009).

4.4. Fungicides

Only the highest concentration of captan killed the springtails in the acute toxicity test. When applying captan (0.3 percent) on apple orchards, Rather and Shah (2010) also reported a short lived effect on collembolan populations in the field. Furthermore, other studies reported that captan is not lethal to earthworms (Anton et al., 1990), even at 1000 mg a.i. kg^{-1} DW in TAS (Alves et al., 2013). We also did not find toxicity of captan on reproduction of *F. candida* at the highest concentration tested (NOEC=1.0 mg a.i. kg^{-1} soil DW). Although Frampton et al. (2006) and Daam et al. (2011) observed higher sensitivity of collembolans towards fungicides than earthworms, concentrations higher than those of our study reduced the reproduction of earthworms at 200 mg kg^{-1} soil DW (Alves et al., 2013). These concentrations were above the PEC, suggesting that the use of seed dressing with captan offers a low risk to these invertebrates. This same fungicide is also known to cause direct (Colinas et al., 1994) and indirect (Ingham et al., 1991) toxicity to nematodes. The last authors found that captan reduces the microbial biomass of soils, and consequently causes effects on mycetophagous nematodes. Collembolans also feed on fungal hyphae (Fountain and Hopkin, 2005) and therefore should be sensitive to this type of indirect effect, but we were not able to test this idea in our study, because a different and uncontaminated food source was provided during the tests.

Except for the highest concentration, the fungicide carboxin+thiram was not lethal to the springtails. For reproduction, no reduction was observed at the concentrations tested. EFSA (2010) also reported that the commercial formulation Vitavax (a.i. carboxin+thiram) does not affect *F. candida* reproduction, even at doses higher than 1000 mg a.i. kg^{-1} DW in artificial soil. The report EFSA (2010) also emphasizes that at these same concentrations this fungicide does not affect reproduction of *Aleochara bilineata* (Coleoptera: Staphylinidae) and *Poecilus cupreus* (Coleoptera: Carabidae). On the other hand, according to Alves et al. (2013), although at higher concentrations than its PEC (0.115 mg a.i. kg^{-1}; Table 1), carboxin+thiram reduced earthworm reproduction (LOEC=25 mg kg^{-1} soil DW).

Both fungicides showed low potential risk for *F. candida*, since there were no effects on reproduction and the impacts on survival only occurred at concentrations higher than their PEC (Table 1). There are other reports of non-toxic effects of the fungicides epoxiconazol++piraclostrobina (1 and 2 L ha^{-1}) and epoxiconazol (0.75 and 1.5 L ha^{-1}) on *F. candida* (Antoniolli et al., 2013). However, Idinger (2002) testing the effects of the fungicide Euparen M WG50 (with the a.i. tolylfluanid) on this species, found the NOEC only at concentrations 80 times below the highest recommended field rate. This suggests that even though the species *F. candida* is not highly sensitive to some fungicides, its susceptibility is dependent on the mode of action of the active ingredient.

4.5. Using ecotoxicological tests for risk prediction

Mortality is one of the factors responsible for a reduction in the number of soil organisms. Declines in collembolan abundance have been observed by several authors (Frampton, 2002; Fountain et al., 2007; Peck, 2009), some of whom have argued that they are associated with a limitation in the decomposition of organic matter (Cortet et al., 2002). However, population reductions are not always associated with lethal effects. San Miguel et al. (2008) reported that under natural conditions some insecticides may pose a low lethal risk to collembolans, but that lower abundance may result from organisms' ability to avoid areas that are seriously contaminated with these substances. Declines in collembolan abundance may also be linked to impacts on reproduction, including the malformation of gametes and embryos, inability to hatch and teratogenic effects. In our study, while all the tested pesticides caused mortality, the effects were significant at concentrations at least 100 times higher (Table 2) than the PEC values (Table 1). This reduces the possibility that population declines of *F. candida* in the field are due to mortality caused by treating seeds with these products. Similar results were found by Coja et al. (2006), who assessed the lethal effect of pesticides on *F. candida* and observed that the concentrations that caused such effects were higher than those normally found in soils and, therefore, were considered not to offer direct risk to these organisms.

The results of our chronic toxicity assays corroborate those of Frampton et al. (2006) and Daam et al. (2011), who report that collembolans were less sensitive to fungicides than to insecticides. However, there are also reports of lower reproduction in collembolans in the presence of fungicides (EFSA, 2010; Idinger, 2002; Jänsch et al., 2006). Reproduction in *F. candida* (EFSA, 2010) was also less sensitive to carboxin+thiram than reproduction of *E. andrei* earthworms (Alves et al., 2013). This difference in sensitivity is likely an exception and should be attributed to the different susceptibility of the organisms to the modes of action of the fungicides. Thus, Frampton et al. (2006) and Daam et al. (2011) reviewed several classes of pesticides and highlights that collembolans were more sensitive than earthworms in acute and chronic tests of a wide variety of pesticides (including biocides, fungicides, herbicides, and insecticides), as we observed in this study.

Although chronic toxicity tests were the most sensitive, detecting effects at concentrations close to those expected in the field (Table 1), the acute toxicity assays represent the endpoint of the worst expected situation and can be useful in the initial screening

of concentrations for the other laboratory tests (e.g. avoidance and reproduction assays). Therefore the use of different/complementary analytical criteria (i.e., more than one type of ecotoxicological test) in the first stage of a pesticide risk assessment for soils increases its sensitivity, since the exposure of organisms to different levels of stress reduces uncertainty regarding the risks these substances pose to soil organisms.

5. Conclusions

All the studied pesticides caused mortality in *F. candida*, but at concentrations exceeding those predicted in the field (PEC). Fipronil and imidacloprid were the most lethal. In the chronic toxicity assays, imidacloprid was the most toxic, followed by fipronil. These pesticides reduced collembolan reproduction at concentrations below and close to their respective PECs. The other pesticides did not interfere with *F. candida* reproduction. The intensity of these effects increased with increasing pesticide concentrations in the TAS. While these results increase our understanding of the toxicity of seed dressing pesticides they must be carefully compared with results obtained for other soil organisms, as well as studies under more realistic conditions (e.g. natural soils), in order to effectively assess the risks posed by these products to the soil fauna. In any case, these results are useful for the revision of the risk assessment of these compounds on the light of the new data requirements for pesticide risk assessment.

Acknowledgments

P.R.L.A. thanks the Graduate Course in Agronomy of UEL (State University of Londrina) and the Soil Microbiology Laboratory of the Soil Science Department at ESALQ/USP (University of São Paulo) for the research opportunity, and wants to express his gratefulness to the Agrisus Foundation for Sustainable Agriculture for a research grant (project: PA-560/09). E.J.B.N.C. thanks for a research grant (No. 307563/2009-0) from the National Council for Technological and Scientific Development (CNPq). E.J.B.N.C. and J.P.S. thank CAPES for support in the cooperation project between University of Coimbra (Portugal) and ESALQ/USP (Brazil) (No. 198/08). The authors thank Tiago Natal da Luz for the technical assistance in the manuscript.

References

Acda, M.N., 2007. Toxicity of Thiametoxam against Philippine subterranean termites. J. Insect Sci. 7 (26), 6.
Achazi, R.K., Rombke, J., Riepert, F., 2000. Collembolen als Testorganismen. In: Heiden, S., Erb, R., Dott, W., Eisenträger, A. (Eds.), Toxikologische Beurteilung von Boden. Spektrum Verlag, Heidelberg, pp. 83–103.
Alves, P.R.L., Cardoso, E.J.B.N., Martines, A.M., Sousa, J.P., 2013. Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. Chemosphere 90, 2674–2682.
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.
Antoniolli, Z.I., Redin, M., de Souza, E.L., Pocojeski, E., 2013. Heavy metal, pesticides and fuels: effect in the population of collembola in the soil. Cienc. Rural 43 (6), 992–998.
Baudet, L., Peske, S.T., 2006. A logística do tratamento de sementes. Seed News 10, 1.
Chelinho, S.L.I., Natal-Da-Luz, T., Domene, X., Nunes, M.E.T., 2012. Integrated ecological risk assessment of pesticides in tropical ecosystems: a case study with carbofuran in Brazil. Environ. Toxicol. Chem. 31, 437–445.
Coja, T., Idinger, J., Blumel, S., 2006. Effects of the benzoxazolinone BOA, selected degradation products and structure related pesticides on soil organisms. Ecotoxicology 15, 61–72.
Coleman, D.C., Crossley, D.A., Hendrix, P.F., 2004. Fundamentals of Soil Ecology, 2nd ed. Elsevier Inc., Athens.
Colinas, C., Ingham, E., Molina, R., 1994. Population responses of target and non-target forest soil organisms to selected biocides. Soil. Biol. Biochem. 26, 41–47.
CONAMA, 2009. Resolução n. 420, (accessed 28.12.09.). ⟨http://www.mma.gov.br/port/conama/legiabre.cfm?codlegi=620⟩. (Conselho Nacional do Meio Ambiente). (accessed 21.01.14.).
Cortet, J., Gillon., D., Joffre, R., Ourcival, J.M., Poinsot-Balaguer, N., 2002. Effects of pesticides on organic matter recycling and microarthropods in a maize field: use and discussion of the litter-bag methodology. Eur. J. Soil. Biol. 38, 261–265.
Coutinho, C.F.B., Tanimoto, S.T., Galli, A., Garbellini, G.S., Takayama, M., Amaral, R.B., Mazo, L.H., Avaca, L.A., Machado, S.A.S., 2005. Pesticidas: Mecanismo de Ação. Degradação e Toxidez. Pesticidas: R. Ecotox. Meio Ambiente 15, 65–72.
Culik, M.P., Zeppelini, D., 2003. Diversity and distribution of Collembola (Arthropoda: Hexapoda) of Brazil. Biodivers. Conserv. 12, 1119–1143.
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.
De Silva, P.M.C.S., Van Gestel, C.A.M., 2009. Development of an alternative artificial soil for earthworm toxicity testing in tropical countries. Appl. Soil. Ecol. 43, 170–174.
Diogo, J.B., Natal-da-Luz, T., Sousa, J.P., Vogt, C., Nowak, C., 2007. Tolerance of genetically characterized *Folsomia candida* strains to phenmedipham exposure – a comparison between reproduction and avoidance tests. J. Soils Sedim. 7, 388–392.
EC, 2013. 284/2013 of 1 March 2013 Setting Out The Data Requirements For Plant Protection Products, in Accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. J.O.L. 93 pp. 1–84. (European Community).
EFSA, 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance carboxin. EFSA J. 8, 1857 (European Food Safety Authority).
FAO, 2012. FAOSTAT – The statistics division, Pesticides trade – import value in Brazil. ⟨http://faostat.fao.org/site/424/DesktopDefault.aspx?PageID=424⟩. (Food Agricultural Organization). (accessed 23.05.13.).
Fountain, M.T., Brown, V.K., Gange, A.C., Symondson, W.O.C., Murray, P.J., 2007. The effects of the insecticide chlorpyrifos on spider and Collembola communities. Pedobiologia 51, 147–158.
Fountain, M.T., Hopkin, S.P., 2005. *Folsomia candida* (Collembola): a "Standard" Soil Arthropod. Annu. Rev. Entomol. 50, 201–222.
Frampton, G.K., 2002. Long-term impacts of an organophosphate-based regime of pesticides on field and field-edge collembola communities. Pest. Manag. Sci. 58, 991–1001.
Frampton, G.K., Jänsch, S., Scott-Fordsmand, J.J., Ro mbke, 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., Rombke, J., de Brito, M.T., Scheffczyk, A., 2008. Effects of three pesticides on the avoidance behavior of earthworms in laboratory tests performed under temperate and tropical conditions. Environ. Pollut. 153, 450–456.
Greenslade, P., Vaughan, G.T.A., 2003. Comparison of Collembola species for toxicity testing of Australian soils. Pedobiologia 47, 171–179.
He, Y., Zhao, J., Zheng, Y., 2012. Lethal effect of imidacloprid on the coccinellid predator *Serangium japonicum* and sublethal effects on predator voracity and on functional response to the whitefly *Bemisia tabaci*. Ecotoxicology 21, 1291–1300.
Heijbroek, W., Huijbregts, A.W.M., 1995. Fungicides and insecticides applied to pelleted sugar-beet seeds – II. Control of pathogenic fungi in soil. Crop. Prot. 14, 367–373.
Heupel, K., 2002. Avoidance response of different collembolan species to Betanal. Eur. J. Soil Biol. 38, 273–276.
Idinger, J., 2002. Laboratory studies to detect effects of selected plant protection products on *Folsomia candida* (Collembola: Isotomidae). J. Plant Dis. Protect 109, 512–529.
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, 1999. Soil Quality – Inhibition of Reproduction of Collembola (*Folsomia candida*) by Soil Pollutants. International Organization for Standardization, Genève (ISO: 11267).
Jackson, G.J., Ford, J.B., 1973. The feeding behaviour of *Phyloseiulus persimilis* (Acarina: Phytoseiidae), particularly as affected by certain pesticides. Ann. Appl. Biol. 75, 165–171.
Jänsch, S., Amorim, M.J., Rombke, J., 2005. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ. Rev. 13, 51–83.
Jänsch, S., Frampton, G.F., Rombke, 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, 3rd ed. Royal Society of Chemistry Information Services, Cambridge.
Kreutzweiser, D.P., Thompson, D.G., Scarr, T.A., 2009. Imidacloprid in leaves from systemically treated trees may inhibit litter breakdown by non-target invertebrates. Ecotox. Environ. Saf. 72, 1053–1057.
Lavelle, P., Decaëns, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerieb, P., Moraa, P., Rossi, J.-P., 2006. Soil invertebrates and ecosystem services. Eur. J. Soil. Biol. 42, 3–15.

Liu, Y.R., Zheng, Y.M., He, J.Z., 2012. Toxicity of profenofos to the springtail, *Folsomia candida*, and ammonia-oxidizers in two agricultural soils. Ecotoxicology 21, 1126–1134.
Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, J., Sieger, E., Walti, M., 2001. The discovery of thiamethoxam: a second-generation neonicotinoid. Pest. Manag. Sci. 176, 165–176.
Marrs, T.T., Ballantyne, B., 2004. Pesticide Toxicology and International Regulation. John Wiley & Sons Ltd, Chichester.
Martikainen, E.A.T., Krogh, P.H., 1999. Effects of soil organic matter content and temperature on toxicity of dimethoate to *Folsomia fimetaria* (Collembola: Isotomiidae). Environ. Toxicol. Chem. 18, 865–872.
Menten, J.O., Moraes, M.H.D., 2010. Tratamento de sementes: histórico, tipos, características e benefícios. Informativo ABRATES 20, 3.
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.
Munkvold, G.P., Sweets, L., Wintersteen, W., 2006. Iowa Commercial Pesticide Applicator Manual. Seed Treatment. Iowa State University, Ames.
Natal-da-Luz, T., Tidona, S., Gestel, C.A.M.V., Morais, P.V., Sousa, J.P., 2009. The use of Collembola avoidance tests to characterize sewage sludges as soil amendments. Chemosphere 77, 1526–1533.
Niemeyer, J.C., Moreira-santos, M., Nogueira, M.A., Carvalho, G.M., Ribeiro, R., Da Silva, E.M., Sousa, J.P., 2010. Environmental risk assessment of a metal-contaminated area in the Tropics. Tier I: screening phase. Ecotoxicology 10, 1557–1571.
NRA, 2001. Evaluation of the New Active Thiametoxam in the Product Cruiser 350FS Insecticide Seed Treatment. (National registration authority for agricultural and veterinary chemicals).
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, Earthworm Acute Toxicity Test. Organization for Economic Cooperation and Development, Paris (OECD No. 207).
Paulsrud, B.E., Martin, D., Babadoost, M., Malvick, D., Weinzierl, R., Lindholm, D.C., Steffey, K., Pederson, W., Reed, M., Maynard, R., 2001. Oregon pesticide applicator training manual. Seed treatment. University of Illinois Board of Trustees, Urbana.
Peck, D.C., 2009. Long-term effects of Imidacloprid on the abundance of surface and soil-active nontarget fauna in turf. Agr. Forest. Entomol. 11, 405–419.
Peijnenburg, W., Capri, E., Kula, C., Liess, M., Luttik, R., Montforts, M., Nienstedt, K., Rombke, J., Sousa, J.P., Jensen, J., 2012. Evaluation of exposure metrics for effect assessment of soil invertebrates. Crit. Rev. Env. Sci. Tec. 42, 1862–1893.
Peveling, R., McWilliam, A.N., 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.
Rather, A.Q., Shah, A.H., 2010. Consequences of pesticide pollution upon the population of Collembola (springtails) in Kashmir apple orchard edaphic ecosystem. J. Ecophysiol. Occup. Health 10, 9–12.
Reynolds, W.N., 2008. Imidacloprid insecticide treatments for hemlock woolly adelgid, *Adelges tsugae annand* (hemiptera: adelgidae), affect a non-target soil arthropod Community Surrounding Eastern Hemlock, *Tsuga canadensis* (L.) Carriere. Dissertation. University of Tennessee.
Rombke, J., Garcia, M.V., 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.
Rusek, J., 1998. Biodiversity of Collembola and their functional role in the ecosystem. Biodivers. Conserv. 7, 1207–1219.
San Miguel, A., Raveton, M., Lempérière, G., Ravanel, P., 2008. Phenylpyrazoles impact on *Folsomia candida* (Collembola). Soil. Biol. Biochem. 40, 2351–2357.
Santos, M.J.G., Soares, A.M.V.M., Loureiro, S., 2010. Joint effects of three plant protection products to the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere 80, 1021–1030.
Six, J., Feller, C., Denef, K., Ogle, S.M., Sa, J.C.D., Albrecht, A., 2002. Soil organic matter, biota and aggregation in temperate and tropical soils – effects of no-tillage. Agronomie 22, 755–775.
Stenersen, J., 2004. Chemical pesticides: mode of action and toxicology. CRC Press LLC, Boca Raton.
Tingle, C.C.D., Rothe, J.A., Dewhurst, C.F., Lauer, S., King, W.J., 2000. Health and Environmental Effects of Fipronil. Pesticide Action Network UK, briefing AI1. ⟨http://www.pan-uk.org/archive/Publications/Briefing/fipronil.pdf⟩. (accessed 1.01.14.).
Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. 45, 247–268.
Waxman, M.F., 1998. Agrochemical and Pesticides Safety Handbook. Lewis Publishers, Boca Raton.