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Environmental Toxicology and Chemistry, Vol. 31, No. 2, pp. 437–445, 2012

Printed in the USA
DOI: 10.1002/etc.738

Hazard/Risk Assessment

INTEGRATED ECOLOGICAL RISK ASSESSMENT OF PESTICIDES IN TROPICAL ECOSYSTEMS: A CASE STUDY WITH CARBOFURAN IN BRAZIL

Sónia Chelinho,*† Isabel Lopes,‡ Tiago Natal-da-Luz,† Xaxier Domene,§ Maria Edna Tenorio Nunes,‖ Evaldo L.G. Espíndola,‖ Rui Ribeiro,† and Jose P. Sousa†

†IMAR–CMA Department of Life Sciences, University of Coimbra, Coimbra, Portugal
‡Departamento de Biologia and CESAM, Universidade de Aveiro, Aveiro, Portugal
§CREAF, Autonomous University of Barcelona, Cerdanyola del Vallès, Barcelona, Spain
‖CRHEA, São Carlos Engineering School, University of São Paulo, São Carlos, São Paulo, Brazil

(*Submitted* 5 *July* 2011; *Returned for Revision* 15 *August* 2011; *Accepted* 5 *October* 2011)

Abstract—The aim of the present study is to contribute an ecologically relevant assessment of the ecotoxicological effects of pesticide applications in agricultural areas in the tropics, using an integrated approach with information gathered from soil and aquatic compartments. Carbofuran, an insecticide/nematicide used widely on sugarcane crops, was selected as a model substance. To evaluate the toxic effects of pesticide spraying for soil biota, as well as the potential indirect effects on aquatic biota resulting from surface runoff and/or leaching, field and laboratory (using a cost-effective simulator of pesticide applications) trials were performed. Standard ecotoxicological tests were performed with soil (*Eisenia andrei*, *Folsomia candida*, and *Enchytraeus crypticus*) and aquatic (*Ceriodaphnia silvestrii*) organisms, using serial dilutions of soil, eluate, leachate, and runoff samples. Among soil organisms, sensitivity was found to be *E. crypticus* < *E. andrei* < *F. candida.* Among the aqueous extracts, mortality of *C. silvestrii* was extreme in runoff samples, whereas eluates were by far the least toxic samples. A generally higher toxicity was found in the bioassays performed with samples from the field trial, indicating the need for improvements in the laboratory simulator. However, the tool developed proved to be valuable in evaluating the toxic effects of pesticide spraying in soils and the potential risks for aquatic compartments. Environ. Toxicol. Chem. 2012;31:437–445. © 2011 SETAC

Keywords—Soil retention function Soil habitat function Laboratory simulator Flume Runoff

INTRODUCTION

Tropical ecosystems constitute major reservoirs of biodiversity that are subject to several threats, including agricultural expansion [1]. This represents a great menace to biodiversity and ecosystem stability. The intensification of agriculture has led to an exponential growth of the demand for pesticides, especially in the tropics [2,3], and Brazil has become, since 2008, the world's top consumer [4]. However, in most tropical countries, this demand for agrochemicals has not been properly accompanied by the development of national legislation regulating their safe use or monitoring their environmental hazards [1,5].

Knowledge of pesticide impact in the tropics is slight compared to that known about its impact in temperate systems, and risk assessment strategies rely mostly on the extrapolation of data from temperate regions [1,6–8]. This approach can lead to biased evaluations of the fate and effects of pesticides because physical, chemical, and biological conditions in the tropics may differ from those in temperate systems [1]. Indeed, the risk of pesticide contamination of environmental compartments—namely, the nonpoint-source contamination of both soil and water resulting from spray drift, volatilization, and mobilization via water through edge-of-field runoff and leaching—may be higher in the tropics [9,10]. In particular, the current severe and unpredictable rainfall [1,3], combined with the existence of extensive systems of irrigation and drainage channels [3,11] and the intensive and/or careless use of pesticides [5,12], can enhance environmental risks. When pesticides are in the tropical environment, their toxicity can be magnified because the number of species affected is usually higher in such highly biodiverse ecosystems [1].

The available data on pesticide fate and effects in the tropics, using relevant local scenarios and test species, are focused mainly on the aquatic compartment [1,5,7]. Several studies have concentrated on rainfall-induced runoff as the main route of pesticide entry into surface waters [10,11,13]. For the soil compartment, although data is scarce (especially effect data), the overall picture is improving, with new input from recent studies that use standardized methods and/or local species [6,8,14–17]. Nevertheless, as emphasized by several authors, more research in tropical ecotoxicology is needed to provide clearer insights into the potential hazards of pesticides in these environments [1,6–8,16].

Further improvements in the ecological risk assessment of pesticides, both in tropical and temperate ecosystems, also include the integration of fate and effect data from both soil and aquatic compartments. Specifically, the risk assessment strategy should encompass the evaluation not only of the pesticide toxic potential of soils as a sink of contaminants for soil organisms, but also of the consequences to aquatic organisms of pesticide mobilization via the water pathway by both leaching and surface runoff. Moreover, the water-mediated transport of pesticides ultimately will determine the quality of water resources for human consumption [9]. Therefore, coordinated approaches are needed that include research on both soil and water protection and that use, whenever possible, cost-effective tools under realistic exposure conditions ([18]; http://tobias-lib.uni-tuebingen.de/volltexte/2004/1320/pdf/SOWA_WS2_Proceedings.pdf).

* To whom correspondence may be addressed (sonia.chelinho@iav.uc.pt).

Published online 8 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

The aim of the present study is to contribute an ecologically relevant assessment of the ecotoxicological effects of applying pesticides in tropical agricultural areas on both soil and aquatic biota. To achieve the main goal, four specific objectives were defined: to evaluate the habitat and retention functions of a tropical soil after carbofuran spraying; to evaluate the potential effects of pesticide spraying on aquatic biota from surface runoff and leaching; to develop tools that are cost-effective and readily amenable to standardization for performing laboratory simulations of pesticide spraying, leaching, and surface runoff; and to evaluate these tools by comparing field and laboratory trial results.

MATERIALS AND METHODS

Field trial

São Carlos (São Paulo [SP], Brazil; −22°10′13.53″, −47°53′58.12″), an area located in the Brazilian sugarcane belt, was chosen for the field trial. The carbamate insecticide carbofuran, widely used in sugarcane plantations [19], was used as a model pesticide.

To simulate realistic exposure scenarios of soil and water contamination, a field with no history of pesticide contamination was selected. The loamy sand soil (5.33 pH, 13.5% organic matter, 79.5% sand, 18.6% silt, 2.17% clay, 0.24% total organic N, 0.687 μg/g total P, and 67.1% water-holding capacity) was analyzed by the Centro de Recursos Hídricos e Ecologia Aplicada (CRHEA, São Carlos, SP, Brazil) according to methods described by Nunes ([20]; http://www.teses.usp.br/teses/disponiveis/18/18139/tde-24012011-140524/es.php). Three days after soil tillage, three realistic scenarios were simulated as described below and illustrated in Figure 1.

Scenario F1: Soil contamination from pesticide spraying. Three parallel strips of land from a flat area (3 × 1 m each and separated by a buffer area of 2 m to avoid cross-contamination) were selected (Fig. 1). Two strips were then sprayed with the insecticide Furadan 350SC, a commercial formulation of carbofuran (FMC; 350 g active ingredient [a.i.]/L). First, the recommended dose (RD) for sugarcane plantations was applied: 5 L/ha, with 1.167 mg a.i./kg soil (dry wt), considering an average soil density of 1.5 g/cm^3 and an incorporation depth of 10 cm. Second, another strip was sprayed with twice the recommended dose (hereafter designated as highest dose [HD]) to mimic pesticide overuse, a common practice among local farmers [21]. Per dose, the required amount of pesticide was diluted in 5 L water collected at a nearby reference lagoon (Represa do Broa, São Carlos, SP, Brazil; −22°10′29.98″, −47°54′5.45″). The third strip of land, the control, was sprayed with the same volume of lagoon water but without the pesticide. After spraying the pesticide or lagoon water, a further 10 L of lagoon water was sprayed to promote insecticide incorporation. After approximately 18 h, soil samples from the top 10 cm were collected for both ecotoxicological tests and chemical analysis.

Scenario F2: Leaching of pesticide-sprayed soil. In a manner similar to scenario F1, three strips of land (4 × 1 m each, located in a flat area and separated by a buffer zone) were used (Fig. 1). For each strip of land, a 30-cm layer of soil was removed, and a plastic lid was laid to create an impermeable stratum to allow leachate collection. This hole was filled with a 10-cm layer of gravel and topped with the soil (20-cm layer) that had been initially removed. Pesticide was applied as described above for scenario F1. One hour after soil spraying, a continuous rain was simulated, causing the soil to reach its maximum water-holding capacity and the water to leach. Approximately 240 L of lagoon water was applied, corresponding to the highest daily precipitation registered in February 2007 (63.9 mm) by the meteorological station of CRHEA, in the surroundings of the field assay. Leachate drained along the plastic lid into a hole dug at the end of the strip, and samples were collected and stored at 14°C until further processing.

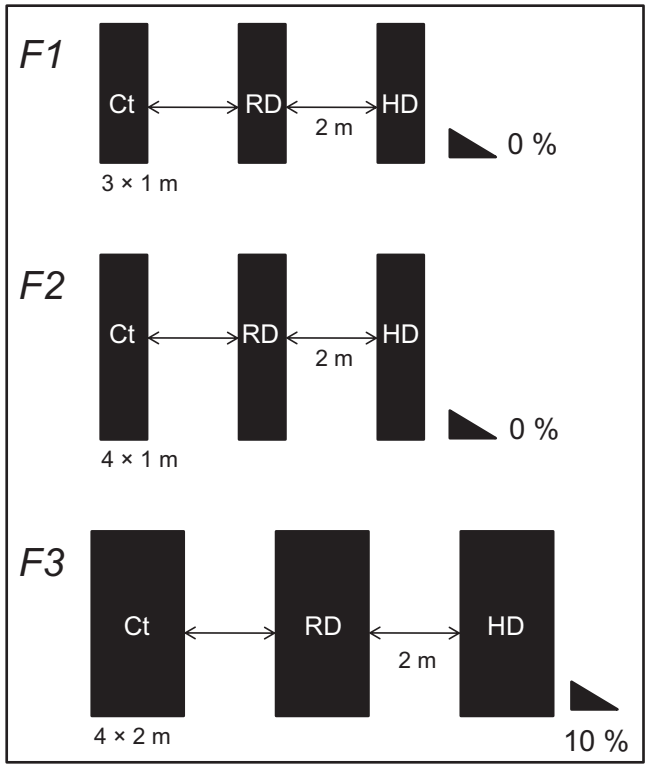

Fig. 1. Plan of the field trial in which three worst-case scenarios of soil contamination with carbofuran were simulated: contamination to soil habitat and retention functions (F1), to aquatic organisms from leaching (F2), and to aquatic organisms from surface runoff (F3). For each scenario the land strips, separated by a 2 m buffer zone, were sprayed with the recommended dose (RD; 5 L/ha) and at a dose twice the RD (highest dose [HD] 10 L/ha) of Furadan (350 g active ingredient/L carbofuran) and lagoon water (Ct). See *Materials and Methods* for details. Slope (%) is given at right.

Scenario F3: Surface runoff of pesticide-sprayed soil. For this particular scenario, the three strips of land (4 × 2 m, separated by the 2-m buffer zone) were located in a 10% slope area. Pesticide application was also performed as described above for scenario F1. One hour after the soil contamination, a heavy rainfall (using ∼190 L lagoon water, corresponding to half of the above-mentioned highest daily precipitation) was simulated, and the runoff water plus the removed soil were collected at a hole dug at the end of the strip and stored at 14°C until further processing.

Simulator trial

A parallel laboratory experiment scheme, with the same soil and the three scenarios described for the field trial, was set up but on a smaller scale. Our aim was to develop and validate a system for performing laboratory simulations of soil contamination and pesticide mobilization via the water pathway. Field scenarios F1, F2, and F3 thus corresponded to the laboratory scenarios S1, S2, and S3. Nine simulators, corresponding to three doses × three scenarios, were built with plastic trays (1.10 × 0.49 × 0.17 m length × width × depth). All the procedures regarding pesticide applications and rainfall simulations

mimicked the field trial. However, the leachate experiment (scenario S2), had a 7-cm layer of gravel plus 10-cm layer of soil. For the runoff experiment (scenario S3) approximately 9 L of the lagoon water was added, and for the leachate experiment the amount of water was approximately 14 L. Perforating the bottoms of the trays facilitated collecting the leachate. For the runoff experiment, plastic containers were adapted to the sloping tray and received the transported soil and water. The slope was similar to that used in scenario F3.

Test organisms

Earthworms (*Eisenia andrei*; Lumbricidae), enchytraeids (*Enchytraeus crypticus*; Enchytraeidae), and collembolans (*Folsomia candida*; Isotomidae) were used as soil test species. These play a crucial role in the soil ecosystem and are used widely in ecotoxicological studies [22]. Standardized procedures to evaluate the habitat function of soils with chemical contamination are available [23–25].

The soil invertebrates were obtained from laboratory cultures at the Laboratory of Soils, University of Coimbra, Coimbra, Portugal. Earthworms and collembolans were maintained as described by Natal-da-Luz et al. [26]. The culture of *E. crypticus* was provided by ECT Oekotoxikologie. In the laboratories of origin, these organisms were maintained in an uncontaminated natural soil at $20 \pm 2^{\circ}C$ with a 16:8-h light:dark photoperiod and were fed weekly with rolled oats. Upon arrival at the CHREA, all organisms were acclimated at $22 \pm 1^{\circ}C$, 12:12-h light:dark for at least one week before beginning the tests. We originally intended to acclimate the organisms and perform the ecotoxicological tests at 24°C, the average of maximum temperatures occurring in the winter season in this area (http://www.cpa.unicamp.br/outras-informacoes/clima_muni_549.html), because the assays were performed in July 2007. However, because of logistical constraints in the laboratory at the time when we had to run the tests, the desired temperature conditions could not be met. Therefore, acclimation was performed under a different temperature (1°C lower) from that used for the tests ($23 \pm 1^{\circ}C$; see below).

The selected aquatic test organism was a native tropical cladoceran (*Ceriodaphnia silvestrii*; Daphnidae). It was chosen for its wide geographical distribution through South America and because it belongs to one of the most sensitive groups of organisms that occupy a central position within the lentic aquatic food chains commonly used to determine toxicity of chemicals and set environmental health standards [27]. In addition, the Associação Brasileira de Normas Técnicas has established a standardized protocol for chronic assays with this species (ABNT NBR 13373 [28]). To allow a direct comparison between lethal and sublethal effects, acute assays were also performed with *C. silvestrii*.

The organisms were continuously reared in CRHEA laboratories in American Society for Testing and Materials (ASTM) soft water (hardness 40–48 mg/L $CaCO_3$ [28]) supplemented with Vitormonio® and Sera Morena (1 ml per 9 ml ASTM), under controlled temperature and photoperiod ($24 \pm 2^{\circ}C$ and 16:8-h light:dark). The medium was changed every 2 d, and organisms were fed daily with *Pseudokirchneriella subcapitata* (10^5 cells/ml).

Ecotoxicological tests

Soil. The soil used for both field and simulator trials was previously defaunated by a freezing (F)–thawing (T) cycle (48-h F: 8-h T: 24-h F) and sieved (5 mm). Control soil was mixed with soil sprayed with HD in different proportions to obtain the following dilution series: 0, 1.25, 2.5, 5, 10, 25, 50, and 100% of HD. The soil organisms were exposed to the different dilutions (with the exception of *E. crypticus*, which was not exposed to the three lowest dilutions), and both mortality and reproduction were assessed. Soil contaminated with the RD of Furadan was also used to compare the performance of organisms with the 50% HD dilution. For all tests, the moisture was adjusted to approximately 50% of the maximum water-holding capacity with deionized water.

Eisenia andrei and *F. candida* tests followed International Organization for Standardization (ISO) guidelines [23,25]. *Enchytraeus crypticus* tests were also based on ISO guidelines [24]; however, the test duration was 28 d and the adults were kept in the vessels until the end of the test. The tests were carried out at $23 \pm 1^{\circ}C$ to simulate winter conditions in the study area, as stated above, and with a 16:8-h light:dark photoperiod. The standard control, Organisation for Economic Co-operation and Development (OECD) soil [29], was used to guarantee test validity.

Water. Lethal and sublethal toxicity assays were performed by exposing 6- to 24-h-old juveniles of *C. silvestrii* to a serial dilution of water samples collected from the runoff and leachate simulations and eluates prepared from soil samples collected from both trials. All dilutions were carried out with the culture medium, ASTM soft water, which was also used as the control for all toxicity assays.

Soil eluates were prepared following German Institute for Standardization guideline DIN 38 414 S4 [30] and stored at 4°C in the dark until use for toxicity testing (within 12–48 h). Lethal assays followed ABNT guidelines [31]. Four replicates were set up per dilution. Runoff and leachates were 0.05, 0.07, 0.10, 0.14, 0.20, 0.28, and 0.39% of RD; the last of these was tested only in the samples obtained from the simulator trial. Eluates of soil samples were 0.07, 0.10, 0.14, 0.20, 0.28, 0.39, 0.55, 0.76, and 1.07% of RD; the last of these was tested only in the samples obtained from the simulator trial. Each replicate contained 10 ml test solution and five neonates of *C. silvestrii*. Mortality was recorded after 24 and 48 h. Assays were performed under controlled temperature and photoperiod ($22 \pm 1^{\circ}C$ and 12:12-h light:dark). As was the case with acclimating the test organisms, we intended to perform the tests at 24°C, but because of similar logistical problems in the laboratory it was not possible to attain this temperature.

Sublethal assays, carried out within one week after the start of the lethal assays, followed ABNT guidelines [28]. Ten replicates were set up per dilution. Based on the results obtained in lethal assays for both laboratory and field simulations, the following range was used in runoff, leachate, and eluate samples: 0.0031, 0.00625, 0.0125, 0.025, 0.05, and 0.1% of RD. Each replicate contained 15 ml test solution and one neonate of *C. silvestrii*. After 9 d of exposure under controlled temperature and photoperiod ($22 \pm 1^{\circ}C$ and 12:12-h light:dark), the time of release of the first brood and the total number of juveniles released per female were recorded. Conductivity, pH, and dissolved oxygen were measured before and after solution renewal and ranged between 7.0 and 8.0 and between 163 and 181 μs/cm, respectively, for the first two parameters; dissolved oxygen was always above (>6.9 mg/L) the critical value for cladocerans (2 mg/L [32]).

Chemical analysis

Soil samples (~150 g) from each dilution were frozen until further processing by the Laboratory of Environmental

Chemistry, IQSC, University of São Paulo. Carbofuran quantifications were performed by liquid chromatography (model SCL-10A-Shimadzu, with ultraviolet detector SPD-10A); confirmation was performed via gas chromatography–mass spectrometry (model QP2010-Shimadzu). The analytes were extracted by a solid-phase extraction method, using C18 columns (U.S. Environmental Protection Agency [U.S. EPA] 3500 and 8270C; methods available at http://www.caslab.com/EPA-Methods/) and ultrasound (EPA 3550B; http://www.trincoll.edu/~henderso/textfi~1/416%20notes/3550b.pdf). Recovery range varied between 78 and 86%. The limit of detection was 5.0 μg/kg (dry wt).

Statistical analysis

Data were analyzed for normality using the Kolmogorov–Smirnov test and for variance homogeneity using Hartley, Cochran, and Bartlett's tests. To evaluate the differences between the organism performance in the control and in the contaminated samples, a one-way analysis of variance (ANOVA) followed by post hoc comparisons with the control (Dunnett's test) was performed in Statistica 7.0 (http://www.statsoft.com/). The lethal concentrations causing 50 and 20% of mortality (LC50s and LC20s; the latter only for tests with *C. silvestrii*) and the respective 95% confidence intervals were calculated by probit analysis with the logarithm of the tested concentrations, in PriProbit software (http://www.ars.usda.gov/Services/docs.htm?docid=11284). The EC50s for reproduction and 95% confidence intervals were calculated using nonlinear regressions [33].

RESULTS

Carbofuran concentrations

Carbofuran concentrations in soil samples are shown in Table 1. A soil dilution gradient was effectively created. With two exceptions, the concentrations in the soil samples from the laboratory trial were slightly lower than those in the field trial (Table 1).

Carbofuran toxicity to soil invertebrates

The effects of carbofuran on the survival and reproduction of the three tested species of soil invertebrates, both in field and in simulator trials, are presented in Figure 2. Derived toxicity data for the same assays are summarized in Table 2. The validity criteria defined by the ISO guidelines [23–25] were achieved in all toxicity tests. The performance of organisms in OECD artificial soil also fulfilled the validity criteria (adult survival: 100, 93, and 98%; average number of juveniles ± standard deviation: 41 ± 10, 584 ± 107, and 494 ± 55; respectively for *E. andrei*, *E. crypticus,* and *F. candida*; data not shown).

Among the three test species, collembolans were the most affected by carbofuran soil contamination. At very low concentrations, 0.460 and 0.429 mg/kg for field and simulator samples, respectively, no adults or juveniles were recorded (Fig. 2, F3 and S3). Reproduction followed a pattern similar to that of mortality, because LC50s and EC50s calculated for each assay are almost similar: 0.057 and 0.073 mg/kg (dry wt) for the field trial and 0.09 and 0.12 mg/kg (dry wt) for the simulator trial (Table 2), respectively, suggesting that, beyond a critical concentration, both survival and reproduction are concurrently impaired.

In contrast, enchytraeids were the least sensitive species. Although reproduction significantly diminished in contaminated soils compared with controls (one-way ANOVA, $p \ll 0.001$; Fig. 2, F2 and S2), the effect was similar in all concentrations tested (Fig. 2, F2 and S2). Despite this, for both field and simulator trials, it was possible to derive the EC50 values of 0.750 and 0.739 mg/kg (dry wt) for the reproduction of enchytraeids exposed to the range of carbofuran concentrations. The effects on adult survival were above the highest concentrations tested (Table 2).

Table 1. Carbofuran concentrations (as milligrams per kilogram of soil dry wt) in the soil samples collected from the field and simulator trials in which the soil was sprayed with two doses of Furadan (the recommended dose [RD; 5 L/ha] and two times the RD [highest dose (HD); 10 L/ha]; 350 g active ingredient/L)[a]

	Carbofuran (mg/kg dry wt)	
Dilutions (% of HD)	Field	Simulator
Control	<0.010	<0,010
1.25	0.027	0.017
2.5	0.039	0.035
5	0.079	0.084
10	0.460	0.191
25	0.400	0.429
50	1.520	1.031
75	1.540	1.170
100	2.460	1.765
RD	1.290	1.178

[a] Soil samples of HD treatment were diluted with uncontaminated soil to obtain the dilutions listed in the table.

Concerning earthworms, mortality was observed at the three highest carbofuran concentrations, ranging from 25 to 47% and 52 to 77% in the contaminated soil collected from field (F1) and simulator (S1) trials, respectively (Fig. 2). Abnormal behavior such as coiling, secretion of mucus, and inability to burrow into the soil was also observed. The reproduction seemed to be stimulated at the two lowest concentrations, especially in the case of dilutions prepared from the field samples (Fig. 2, F1). Despite the higher mortality observed in the test performed with samples from the simulator trial, the effects on reproduction were more pronounced in the test performed with samples from the field trial. In the latter the EC50 value was 0.08 mg/kg (dry wt), whereas for the simulator it was 0.30 mg/kg (dry wt) (Table 2).

Although the same carbofuran dilutions were used in both assays, toxicity was somehow higher in soil samples from the field trial. For instance, the EC50 values for reproduction in soil dilutions from the field assay were approximately 3.8- and 1.6-fold lower, respectively, for *E. andrei* and *F. candida* (Table 2).

Carbofuran toxicity to cladocerans

The mortality of *C. silvestrii* in the ASTM controls and in the uncontaminated water samples was lower than 10%, fulfilling the validity criteria defined by the guidelines for lethal assays [31]. In the sublethal tests, the average number of neonates produced by females at the end of the 9-d assay was slightly below the value indicated by the ABNT guideline (15 neonates per female; 14.6 ± 4 and 12.8 ± 3 for the tests with simulator and field samples, respectively; data not shown). These results probably were due to the lower temperature (22 ± 1°C) of the room in which tests were conducted compared with that indicated in the guideline (24 ± 1°C). In fact, temperature is a factor that strongly influences the age at first reproduction, namely, for *C. silvestrii* (see Fonseca and Rocha [34], and references therein).

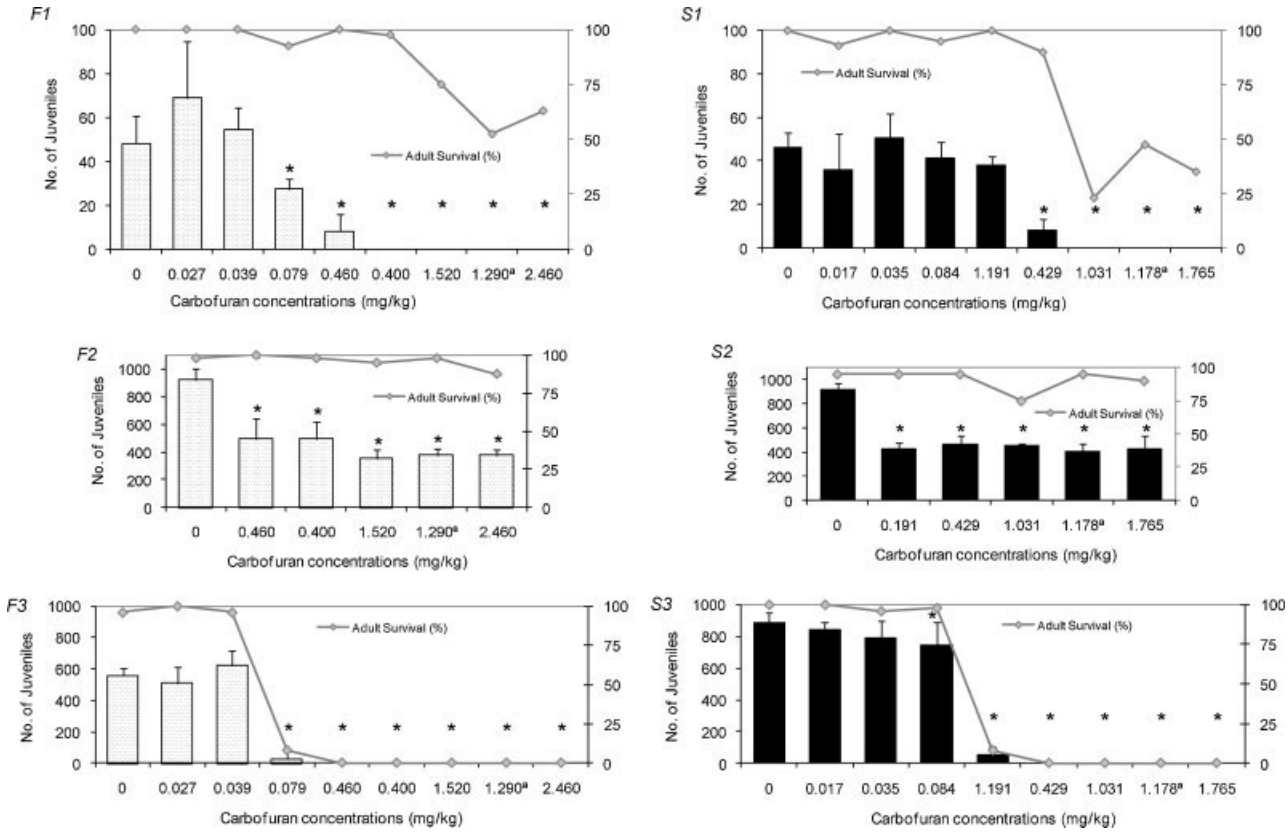

Fig. 2. Adult survival (gray lines) and juvenile production (bars; average ± standard deviation) of three soil invertebrate species, (F1, S1) *Eisenia andrei*, (F2, S2) *Enchytraeus crypticus*, and (F3, S3) *Folsomia candida*, exposed to soil sprayed with carbofuran at the recommended dose (RD; 5 L/ha) and at a dose twice the RD (highest dose [HD]; 10 L/ha) of Furadan; 350 g active ingredient/L) in the field (F) and simulator (S) trials. See text for details and Table 1 for correspondence with Furadan dilutions. [a]Concentration corresponding to RD; [*]Number of juveniles significantly lower than control (one-way analysis of variance, Dunnett's test, $p < 0.05$).

The lethal effects of carbofuran on *C. silvestrii* in eluate, leachate, and runoff samples collected from contaminated soil are shown in Figure 3, and the derived toxicity values for lethal and sublethal tests are presented in Table 3. Results revealed that this cladoceran species is extremely sensitive to carbofuran contamination. From the three types of aqueous samples, high lethality was observed for runoff, followed by leachates and eluates (Fig. 3). The LC20s derived from runoff dilutions were, for both trials, below 0.07% RD (Table 3), and at the highest dilutions (0.28% and 0.39% RD), after 48 h of exposure, 80% or more of the cladocerans died (Fig. 3, F2 and S2).

The LC20s and LC50s computed for the runoff samples indicated that these samples were twice as toxic as the leachate samples (Table 3). Despite this, median lethal effects occurred at dilutions of only 0.25 and 0.53% RD for the field and simulator trials, respectively; the latter value was extrapolated.

In the assays performed with eluate samples, toxicity was much lower because less diluted samples induced lower mortality (at the highest dilutions, 0.76 and 1.07% of RD for the field and simulator trials, respectively, the percentage of dead cladocerans were ≤30% after 24 h [Fig. 3, F1 and S1] and ≤45% after 48 h of exposure [Fig. 3, F2 and S2]). Although for these tests the LC50s were extrapolated from the regression model and thus comparisons should be made cautiously, the LC20s point to a significant toxicity at dilutions lower than 1% RD (Table 3).

As with the tests with soil organisms, a higher lethal toxicity was observed for samples obtained from the field trial. This was more evident in the derived LC50s, which varied by a factor of

Table 2. Toxicity of carbofuran to *Eisenia andrei*, *Enchytraeus crypticus*, and *Folsomia candida* exposed to soil contaminated with the recommended dose (RD; 5 L/ha) and two times the RD (highest dose [HD]; 10 L/ha) of Furadan (350 g active ingredient/L) in the field and simulator trials (see text for details)[a]

	Carbofuran toxicity (mg/kg dry wt)			
	Survival (LC50)		Reproduction (EC50)	
Organism	Field	Simulator	Field	Simulator
E. andrei	3.13 (2.32–5.69)	0.75 (0.64–0.87)	0.08 (0.06–0.11)	0.30 (0.22–0.41)
E. crypticus	>2.46	>1.77	0.75 (0.41–1.36)	0.74 (0.38–1.44)
F. candida	0.057 (0.053–0.063)	0.09 (0.07–0.10)	0.073 (0.069–0.078)	0.12 (0.10–0.14)

[a] Toxicity data include median lethal concentration (LC50; effects on survival) and median effective concentration (EC50; effects on reproduction) values as well as the 95% confidence intervals (in parentheses). Data are expressed in terms of carbofuran concentrations (as milligrams per kilogram soil dry wt; see Table 2 for correspondences with Furadan dilutions).

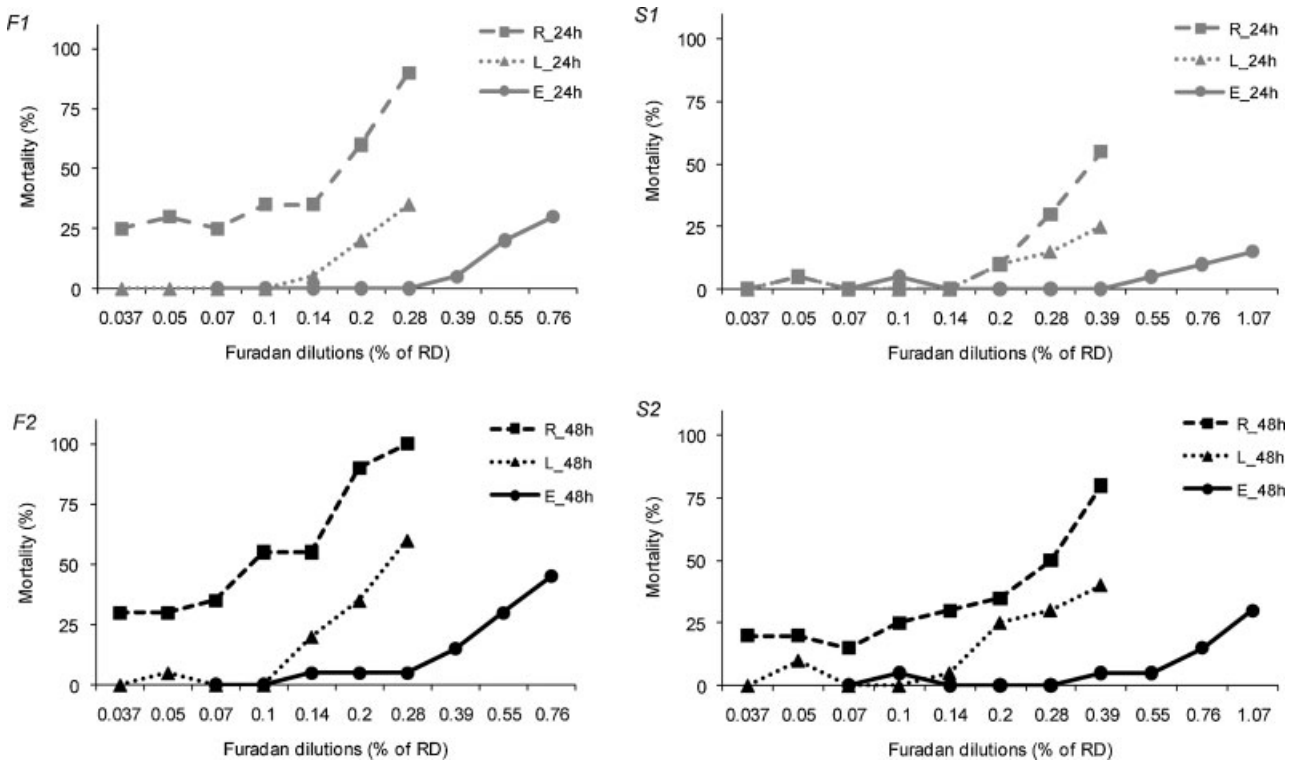

Fig. 3. Mean neonatal mortality of *Ceriodaphnia silvestrii* exposed for 24 h (F1, S1) and 48 h (F2, S2) to several dilutions of runoff (R), leachate (L), and eluate (E) samples collected following soil spraying with the recommended dose (RD 5 L/ha) of Furadan (active ingredient carbofuran, 350 g/L), in the field (F) and simulator (S) trials (see text for details).

2.9, 2, and 3 for the tests with runoff, leachate, and eluate samples, respectively (Table 3).

In the chronic toxicity tests, the calculation of EC50s was not possible because, except for 0.1% RD of the field trial, none of the dilutions caused reproduction impairment equal to or above 50%, except for 0.1%. Despite this, for runoff samples, the highest dilution tested (0.1% RD) caused a significant decrease in reproduction compared with the control in both field and simulator trials (one-way ANOVA; Dunnett's test, $p = 0.001$; data not shown). Indeed, the reproduction of cladocerans was on average 60 and 38% lower at this dilution relative to the control for the field and simulator trials, respectively (data not shown).

DISCUSSION

Carbofuran concentrations

The measured soil carbofuran concentrations in RD and HD treatments at the beginning of the trials were close to the expected values (1.167 and 2.33 mg/kg [dry wt] for RD and HD, respectively; see *Materials and Methods* and Table 1). The higher concentrations found in the samples from the field trial were unexpected. Indeed, pesticide losses in the field are expected to be higher than in a laboratory context. Nevertheless, in both trials, the pesticide spraying was performed in the late afternoon and very close to the soil surface to minimize pesticide dissipation by volatilization and/or spray drift.

Carbofuran toxicity to soil organisms

The three soil invertebrate species used in the present study showed a markedly different sensitivity to carbofuran. The highest toxicity, registered in *F. candida* tests, was probably related to the insecticidal properties of carbofuran. The derived toxicity parameters were clearly below the recommended doses, for both field and simulator trials. Indeed, EC50s and LC50s corresponded, at most, to 9% and 7% of the concentration found in the RD, respectively (Tables 1 and 2), anticipating a serious

Table 3. Toxicity of Furadan (active ingredient carbofuran, 350 g/L) to *Ceriodaphnia silvestrii* exposed to eluate, runoff, and leachate samples collected following the soil spraying with the recommended dose (RD; 5 L/ha) from the field and simulator trials (see text for details)[a]

	Furadan toxicity (%RD)					
	LC20 (48 h)		LC50 (48 h)		EC50 (9 d)	
Sample	Field	Simulator	Field	Simulator	Field	Simulator
Runoff	0.04 (0.05–0.02)	0.06 (0.09–0.03)	0.08 (0.10–0.07)	0.24 (0.44–0.17)	>0.05 < 0.1	>0.1
Leachate	0.15 (0.18–0.12)	0.21 (0.30–0.15)	0.25 (0.36–0.21)	0.54[b] (1.44–0.35)	>0.1	>0.1
Eluate	0.43 (0.57–0.33)	0.96 (2.65–0.64)	0.89[b] (1.80–0.65)	2.76[b] (30.17–1.40)	>0.1	>0.1

[a] Toxicity data include LC20, LC50 (effects on survival), and EC50 (effects on reproduction) values as well as the 95% confidence intervals (in parentheses). Data are expressed in terms of percentage of RD. LC = lethal concentration; EC = effective concentration.
[b] Extrapolated values.

risk of carbofuran applications for nontarget arthropods if a significant amount of the pesticide reaches the soil surface. The LC50s derived in the present study (Table 2) were within the range of the values (0.06–0.15/kg) reported by Van de Plassche [35].

Earthworms were the second most affected group in the present study. As an inhibitor of AChE activity, carbofuran provokes neurotoxic effects, which are more or less reversible, depending on the amount and time of exposure [36]. Toxicity was higher in the field trial, except for earthworm survival, which was less affected than in the tests with samples from the simulator trial (Fig. 2, F1). Despite this difference, the abnormal behavior observed in earthworms recovered from the highest carbofuran concentrations indicates that, for both assays, these organisms would probably die within a short period of time. Van Gestel [37] reviewed the acute effects of carbofuran on earthworms in field and laboratory tests. The author reported 14-d LC50s in artificial soil for *E. andrei* between 5 and 10 mg/kg, which are higher than those obtained in our study. Indeed, LC50s varied between 3.13 and 0.75 mg/kg (dry wt) for the field and simulator assays, respectively (Table 2). This difference can be related to different pedological properties of the test soils, because they can strongly modify chemical toxicity [38]. In field studies, Van Gestel [37] reported that estimated soil carbofuran concentrations of 1.4 to 16 mg/kg caused a reduction in earthworm population equal or higher than 50%. These results are slightly above the range of concentrations measured for the RD of the commercial formulation used in the present study, 1.29 and 1.18 mg/kg (dry wt) for the field and simulator trials, respectively (Table 1).

Recently, aiming to generate more data on tropical soil toxicity, De Silva and colleagues [6,14,15] conducted a series of tests with tropical and temperate earthworm species, including *E. andrei*, in pesticide-contaminated soils (OECD artificial and modified soil plus tropical and temperate natural soils). When exposing *E. andrei* to standard OECD and two modified standard soils (alternatives for the tropics) plus two natural soils (representing temperate and tropical soils) contaminated with carbofuran, both LC50s and EC50s were higher than those obtained in the present study. Indeed, the lowest LC50 and EC50 were respectively 8.46 and 0.6 mg/kg [6,14], whereas in our study the same toxicity parameters for the field (F) and simulator (S) assays were 3.13 (F) 0.75 (S) and 0.08 (F) 0.30 (S) mg/kg (dry wt) (Table 2). Again, the different soil properties of the test substrates [38], namely, the higher pH, as well as the pesticide formulation used [6] could explain these variations. The latter study used pure carbofuran, whereas in our study a commercial formulation was applied. In another study, the same authors found higher toxicity of the formulated carbofuran compared to the pure substance for the tropical earthworm *Perionyx excavatus* [15]. The avoidance behavior of *E. andrei* was also found to be stronger toward soil contaminated with a commercial formulation of the herbicide penoxsulam compared with the pure chemical [39]. Commercial formulations probably contain other chemical agents that could either enhance the toxicity of the pesticide or that are themselves toxic. The higher toxicity of pesticide formulations relative to pure substances has been documented for other test species (see references cited by De Silva et al. [15]). Data from *E. crypticus* tests suggest that the application of the carbofuran formulation within the recommended doses does not seem to be lethal, because with two exceptions adult survival was always higher than 90% in all concentrations tested (Fig. 2, F2 and S2). Despite this, the reproductive potential of the population may be endangered, because the reproduction in the lowest concentration tested, corresponding to 10% HD, was about 45 and 30% less than in the controls for the tests with field and simulator samples, respectively (Fig. 2, F2 and S2, and Table 1). Moreover, in the tests with samples from both the field and simulator trials, carbofuran concentrations of approximately 0.7 mg/kg (dry wt) caused a decrease of 50% in reproduction (Table 2). Data from field and semifield tests on carbamate toxicity to enchytraeids suggest that these organisms are sensitive to some carbamates and indifferent to others [40].

Carbofuran toxicity to cladocerans

Carbamate insecticides are known to be highly toxic to aquatic organisms, and aquatic crustaceans are particularly endangered [41,42]. Indeed, carbofuran was much more toxic for cladocerans than for soil organisms. For example, in the present study, median lethal values for *F. candida*, the most affected soil species, corresponded to approximately 5% RD (Tables 1 and 2), whereas, for *C. silvestrii*, in tests with runoff samples, the calculated values were below 0.25% RD (Table 3).

Among the three water samples tested, runoff was clearly the most toxic. According to the literature, pesticide losses by runoff seem to be the most important exposure route of non-point-source pollution of surface waters [10], and peaks of concentration and toxicity have been reported to occur after applications and/or rainfalls [10,11,42,43]. For example, high concentrations of carbofuran, ranging from 0.010 from 1.823 mg/L and its metabolites have recently been detected in water samples from Kenya during the rainy season [44]. Schulz [10] estimated that 1 to 10% of the total amount of applied pesticide, or even more if severe rainstorms follow pesticide application, is lost by edge-of-field runoff.

The higher toxicity observed in cladocerans exposed to leachate dilutions is consistent with the high leaching potential of carbofuran [45,46]. This behavior is due to its high water solubility (322 mg/L), low sorption ($K_{OC} \sim 23.3$ ml/g), and moderate persistence (soil median degradation time (DT50) ~ 29 d; aqueous hydrolysis DT50 at 20°C and pH 7 ~ 37 d; http://sitem.herts.ac.uk/aeru/iupac/118.htm). Martins et al. [45] calculated that approximately 6% of the total amount of carbofuran applied to the surface of a tropical soil is leached below 50 cm. The lower toxicity of leachate dilutions, compared with runoff, may be related to the adsorption of some carbofuran to the soil organic matter during its percolation trough the soil profile. Despite this contribution of soil organic matter to pesticide sequestration, the sandy texture seems to have mitigated this effect and increased carbofuran mobility [46].

Eluates were by far the least toxic samples: the calculated LC20s and LC50s were at least 11 times higher than those derived for tests with runoff dilutions (the most lethal; Table 3). The time involved in the eluate preparation, consisting of 12 h shaking plus 12 h of rest, could have contributed to some carbofuran degradation. Also, the basic conditions (average pH values of 7.8 for field and 7.7 for laboratory dilutions; data not shown) seem to favor carbofuran hydrolysis [41]. The soil eluates are recommended by ISO guidelines [47] as a useful method to evaluate the soil retention function and leaching potential and, thus, the risks for organisms exposed to pesticide mobilization via water. However, results from the present study clearly showed that eluates are not good indicators of the possible contamination of both ground and surface waters. Therefore, it is strongly recommended that, in assessment of the potential hazard of pesticide-contaminated soils to aquatic

systems, the testing strategy should include tests with runoff and leachate samples.

In general, the range of dilutions tested did not cause effects on reproduction of *C. silvestrii*. Because the dilutions were defined after taking into account the results from lethal tests, these unexpected results probably are due to the rapid carbofuran degradation in the water samples during the gap of time between the beginning of the lethal and the sublethal tests.

In summary, results from the present study clearly show that the application of this insecticide at the recommended doses poses a serious risk for the aquatic ecosystem, because in the runoff and leachate samples collected from a worst-case-scenario simulation, dilutions of less than 1% RD were clearly toxic to a cladoceran species. Nevertheless, in a real contamination scenario, several variables may increase or decrease the threats posed by carbofuran. Indeed, when entering a water system, the carbofuran concentration in runoff and leachate would be more or less reduced, depending on factors such as the size of the waterway, the flow, or other physical conditions that affect bioavailability to aquatic organisms (e.g., amount of dissolved particles, dissolved organic carbon, temperature, pH) and, thus, the exposure level [43]. However, intensive and/or excessive applications of the pesticide, as well as the severe precipitation occurring in the tropics, might amplify the risk [11].

The hazardous carbofuran concentration affecting 5% of the aquatic species (HC5) in single-species acute tests estimated by Maltby et al. [48] was 0.2 μg/L. With the exception of the European Union, which established a maximum level of 0.1 μg/L for each pesticide [49], this value is clearly below the guidelines for carbofuran concentrations in drinking water established by several countries, which vary from 5 μg/L in Australia to 90 μg/L in Canada (World Health Organization, 2004; http://www.who.int/water_sanitation_health/dwq/chemicals/carbofuran.pdf), thus pointing to a hazardous potential of legally accepted carbofuran concentrations at least for some aquatic organisms. In addition, the disappearance of natural grazers such as zooplankton due to lethal insecticide concentrations may indirectly alter the abundance and composition of the phytoplankton community, eventually leading to algal blooms [50]. Moreover, the lack of food for zooplankton predators, such as fish larvae, may also disturb local food webs.

Toxic balance of field and simulator trials

The higher carbofuran toxicity observed in the field versus the simulator trial is consistent with the higher measured carbofuran concentrations in the former assay (Tables 1 and 2). Moreover, the toxicity parameters affecting the organisms' responses, for both soil and aquatic species, in the two sets of field and simulator trials varied from 1 to 3.8 times (Tables 2 and 3). Despite the parallel methodologies followed in both trials, these deviations seem to be acceptable as uncertainties associated with, for example, the different application scales and physical conditions. Römbke and Moltmann [51] inferred that results from the same test system, using the same chemical but performed in different laboratories, could have results with a deviation factor of up to 10. Thus, the system developed to simulate pesticide applications in the laboratory, allowing the further evaluation of toxic effects to both soil and aquatic organisms, proved to be a good surrogate of expensive and complex field studies. Still, to continue with a standardization process, some improvements are most advisable. These would include changing from plastic to steel to avoid possible pesticide adsorption, or adding features that would facilitate testing under variable slopes and collection of aqueous samples.

CONCLUSION

The field application rates of the carbofuran formulation were hazardous to soil organisms, indicating deleterious effects on habitat function. The aquatic cladocerans were most affected by the carbofuran applications, suggesting that the soil retention function is low. Among the three tested soil species, the most affected group were collembolans, followed by earthworms and enchytraeids. The present study also showed that, in the aquatic compartment, major risks of carbofuran contamination and toxicity arise from surface runoff inputs to adjacent water systems resulting from heavy rainfalls after pesticide application. In fact, from the three aqueous samples tested, the highest lethal toxicity to *C. silvestrii* was found with runoff dilutions. Although at least half as toxic as runoff samples, the high mortality observed in leachate samples pointed to increased risks of groundwater contamination. Soil eluates were by far the least toxic samples, showing the need to include tests with runoff and leachate samples in the test strategy. Moreover, because they represent realistic routes of exposure of aquatic organisms to pesticide contamination, the ecological relevance of the gathered data would be increased.

A good consistency was found between the toxicity results of tests performed with samples collected from field and simulator trials. Thus, even though improvements are still needed, the laboratory simulator proved to be a valuable tool for evaluating the toxic effects of pesticide spraying in soils and the potential risk to aquatic organisms resulting from runoff and leaching.

Acknowledgement—The present study was sponsored by the Fundação para a Ciência e a Tecnologia, Portugal, and Fundo Social Europeu and Programa Operacional Potencial Humano funds (Programa Ciência 2007) through doctoral and postdoctoral grants to S. Chelinho and I. Lopes (SFRH/BD/27719/2006 and SFRH/BPD/7192/2001).

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