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ECOTOXICOLOGY IN TROPICAL REGIONS

Acute and chronic toxicity of diuron and carbofuran to the neotropical cladoceran *Ceriodaphnia silvestrii*

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Abstract In order to contribute to the increase of the body of knowledge on the sensitivity of tropical indigenous species to pesticides, acute and chronic toxicity tests were conducted with the neotropical cladoceran *Ceriodaphnia silvestrii*. Tests were carried out with the active ingredients diuron and carbofuran and one of their commercial formulations, the Diuron Nortox® 500 SC and the Furadan® 350 SC, respectively. For carbofuran, the active ingredient was more toxic than the commercial product, whereas for diuron, the commercial product appeared more toxic. In addition, hormetic effects on fertility were recorded for intermediate diuron concentrations. Acute and chronic toxicity data indicated that *C. silvestrii* was among the most sensitive invertebrate species for both test compounds. Based on concentrations measured in Brazilian water bodies, these compounds represent ecological risks for causing direct and indirect toxic effects on *C. silvestrii* and other aquatic organisms. Our results support previous claims on the advantages of using native species to better tune ecological risk assessment of chemicals in tropical ecosystems.

Keywords Herbicide · Insecticide · Active ingredients · Commercial formulations · Microcrustacean · Tropics · Aquatic ecotoxicology · Species sensitivity distribution

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Introduction

Pesticides are known for their favorable cost-benefit relationship in pest, disease, and weed control and subsequently increasing crop productivity. However, as these compounds are not selective to their target organisms, they may cause deleterious toxic effects on beneficial organisms and ecosystem functioning (Schreinemachers and Tipraqsa 2012). Following the Green Revolution, the use of pesticides in tropical agricultural systems has increased considerably (Silva and Van Gestel 2009). This is also the case for Brazil, which became the world's largest pesticide consumer in 2008 (Rigotto et al. 2014), and excessive consumption has remained in ascension since then (IBAMA 2014).

Due to relatively high mobility of pesticides in tropical soil as a result of frequent torrential rains and intensive irrigation practices, many agrochemicals are transported through leaching and runoff to surface water and groundwater (Wightwick and Allinson 2007). Diuron and carbofuran, for example, have often been reported in Brazilian water bodies (e.g., Carbo et al. 2008; Caldas et al. 2011; Ribeiro et al. 2013; Loro et al. 2015) and elsewhere in the world (e.g., Faggiano et al. 2010; Kaonga et al. 2015; Masiá et al. 2015; Papadakis et al. 2015).

Diuron (*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea) is a herbicide that inhibits photosynthesis by blocking the electron transfer at the level of photosystem II in photosynthetic microorganisms and plants (Giacomazzi and Cochet 2004).

Despite its target action on weeds, diuron can also cause toxic effects on heterotrophic nontarget organisms (invertebrates, amphibians, and fishes) through different modes of action, such as acetylcholinesterase (AChE) inhibition (Bretaud et al. 2000; Ahmed et al. 2012), teratogenicity (Lazhar et al. 2012), endocrine disruption (Noguerol et al. 2006; Orton et al. 2009), and immunotoxicity (Luna-Acosta et al. 2012). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-*N*-methylcarbamate) is an insecticide, acaricide, and nematicide with AChE-inhibiting mode of action (Pessoa et al. 2011). This compound is considered to be very toxic to birds, bees, and aquatic animals such as invertebrates and fish (Ibrahim and Harabawy 2014).

Most studies into the toxicity and risk evaluation of pesticides for aquatic ecosystems have been conducted in temperate region countries (Lacher and Goldstein 1997; Racke 2003; Daam and Van den Brink 2010). Consequently, aquatic risk assessments in tropical countries often rely on toxicity data from temperate species (Kwok et al. 2007), such as the temperate standard invertebrate species *Daphnia magna* and *Ceriodaphia dubia*, although the fate and effects of pesticides may differ between climatic regions (Daam and Van den Brink 2010). This implies little ecological realism of current tropical environmental risk assessments, and the estimated risk based on temperate taxa may not reflect that occurring under tropical conditions (Garcia 2004; Silva and Van Gestel 2009). Studies using native tropical species have shown that these can be more sensitive than test organisms of temperate environments (e.g., Freitas and Rocha 2012; Moreira et al. 2014). Moreover, there is always the risk of accidently introducing temperate exotic species in tropical ecosystems when testing such species in laboratories situated in the tropics (Freitas and Rocha 2011). Therefore, there is a growing need to obtain ecotoxicological data under tropical conditions using native indigenous test organisms for the environmental risk assessment of chemicals.

As relatively little is known about the sensitivity of tropical species, compared to those from temperate regions, it becomes indispensable to obtain data through ecotoxicological tests adapted to tropical conditions (Lacher and Goldstein 1997; Daam and Van den Brink 2010). Several studies have proposed tropical cladocerans for use in ecotoxicological tests, e.g., *Ceriodaphnia cornuta* and *Daphnia lumholtzi* (Vietnam; Bui et al. 2016), *Moina micrura* (Burkina Faso (Leboulanger et al. 2011) and Thailand (Iwai et al. 2011)), *Moinodaphnia macleayi* (Northern Australia; Van Dam et al. 2004), *Diaphanosoma brachyurum* (Brazil; Lopes et al. 2007), *Pseudosida ramosa* (Brazil; Freitas and Rocha 2012), and *Ceriodaphnia silvestrii* (Brazil; Casali-Pereira et al. 2015). However, only few pesticides have been tested using these organisms, so the sensitivity of these tropical cladocerans for a wide range of pesticides, and hence their potential for use as test species in tropical environmental effect assessment, remains largely unknown.

The aim of the present study was to evaluate the toxicity of the active ingredients diuron and carbofuran and their commercial formulations to the Neotropical cladoceran *C. silvestrii*. This species presents a wide geographic distribution in South America, has a short life cycle and is easy to maintain and culture in the laboratory, belongs to one of the most sensitive group of organisms for a variety of toxic substances, and occupies a key role in aquatic food chains (Fonseca and Rocha 2004). Methods for laboratory cultivation and toxicity testing have previously been developed (Fonseca and Rocha 2004; ABNT 2005; Santos et al. 2006). To increase our understanding of the sensitivity of this species to pesticides, the toxicity of diuron and carbofuran to *C. silvestrii* was evaluated through acute and chronic toxicity tests to determine 48-h EC_{50} (immobility) and 8-day no observed effect concentration (NOEC) (reproduction) values, respectively. We also evaluated the sensitivity of *C. silvestrii* relative to temperate species through species sensitivity distributions and discussed the importance of using the native species in tropical risk assessments.

Materials and methods

Test organism and culture conditions

The cladoceran *C. silvestrii* Daday, 1902 (Crustacea, Cladocera, Daphnidae) was initially isolated from the Lobo-Broa Reservoir, Itirapina, SP, Brazil, and has been kept in stock cultures for more than 4 years at the Ecotoxicology Laboratory of the Federal University of São Carlos (Brazil).

The *C. silvestrii* cultures were kept under controlled temperature (25 ± 1 °C) and photoperiod (12:12-h light/dark) in reconstituted water with pH 7.0–7.6, conductivity of 160 μS cm^{-1}, and hardness of 40–48 mg L^{-1} (as $CaCO_3$), as recommended by the Brazilian Association of Technical Standards (ABNT 2005). The organisms were fed daily with the chlorophycean algae *Raphidocelis subcapitata* (10^5 cells mL^{-1}), which was grown in CHU-12 medium (Müller 1972), and a suspension containing yeast (0.5%) and fermented fish food (0.5%) was added as a food supplement (1 mL L^{-1}) (ABNT 2005).

Chemicals and test solutions

Diuron (Chemical Abstracts Service (CAS) number 330–54-1) and carbofuran (CAS number 1563–66-2), both of high purity (≥98%, analytical standard), were purchased from Sigma-Aldrich. The composition of the commercial formulation Diuron Nortox® 500 SC (purchased from Nortox S/A, Brazil) is 50% *m*/*v* of active ingredient (a.i.) (69.4% *m*/*v* of inert ingredients) and that of Furadan® 350 SC (purchased from FMC, Brazil) is 35% *m*/*v* of active ingredient (65% *m*/

v of inert ingredients). The stock solutions of commercial diuron (100 mg a.i. L^{-1}), commercial carbofuran (100 mg a.i. L^{-1}), and carbofuran (100 mg a.i. L^{-1}) were prepared by dilution of a specific amount of each compound in distilled water immediately prior to the tests, with exception of diuron, which was prepared in analytical-grade acetone (C_3H_6O; LabSynth) due to its low solubility in water (42 mg L^{-1} at 20 °C; Giacomazzi and Cochet 2004). The same stock solution concentrations were used in both acute and chronic toxicity tests. Nominal concentrations of each compound tested were obtained by dilution of the stock solution in culture medium (reconstituted water).

Chemical analysis of the pesticides

To confirm the nominal concentrations used in the tests, initial test concentrations were analyzed using an Agilent Technologies Series 1200 high-performance liquid chromatography (HPLC) (Waldbronn, Germany), equipped with a diode array detector (DAD). The chromatographic analytical conditions were based on those described by Mansano et al. (2016), Agilent Zorbax ODS C18 column (250 × 4.6 mm × 5 μm) (Agilent Technologies, USA), oven temperature at 25 °C, isocratic mobile phase of acetonitrile and Milli-Q water (70:30, *v*/*v*), injection volume of 20 μL, flow rate of 1.0 mL min^{-1}, and run time of 6 min. Analyses were carried out in three replicates. Based on absorbance signals observed in the DAD spectrum of the standard solutions, diuron and carbofuran were detected and quantified at 254 and 280 nm, respectively. The retention times found for carbofuran and diuron were 3.5 and 4.2 min, respectively. The diuron test solutions were analyzed by direct injection in HPLC-DAD, while those of carbofuran were concentrated by solid phase extraction (SPE) prior to injection in HPLC-DAD. The SPE performed was adapted from the method described by Cappelini et al. (2012). First, the Chromabond® C18ec cartridges (6 mL, 500 mg; Macherey-Nagel, Duren, Germany) were conditioned with 10 mL acetonitrile followed by 10 mL Milli-Q water, and then, 200 mL sample was passed through the cartridges under vacuum. The carbofuran analyte was eluted with 5 mL of acetonitrile and then subjected to evaporation with nitrogen and reconstituted in 0.5 mL acetonitrile for chromatographic analysis. This procedure was conducted in triplicate for each carbofuran test concentration. The detection and quantitation limits and linear correlation coefficients of the curve, precision, accuracy, and recovery for the carbofuran and diuron analyses are shown in Supplementary Material Table S1.

Acute and chronic toxicity tests

The acute and chronic toxicity tests followed the protocols issued by ABNT (2004, 2005). Based on the results from preliminary tests, the range of concentrations for each compound was established and acute toxicity tests were carried out at the following nominal concentration ranges: 2000 to 32,000 μg L^{-1} of diuron and 400 to 2000 μg L^{-1} of diuron dosed as Diuron Nortox® 500 SC, 0.16 to 2.60 μg L^{-1} of carbofuran, and 0.60 to 3.93 μg L^{-1} of carbofuran dosed as Furadan® 350 SC.

In the acute toxicity test, four replicates were used for each of the five pesticide treatments and the control. Each replicate consisted of a nontoxic polypropene plastic cup containing five neonates (6–24 h old) in 10 mL test solution or 10 mL reconstituted water (control). In the case of active ingredient diuron, it was necessary to add a solvent control (reconstituted water + acetone) with a final concentration of 0.01% acetone (*v*/*v*). The experiments were maintained at 25 ± 1 °C, without addition of food and in darkness. After 48-h exposure, the organisms were observed under a stereomicroscope and the number of immobile individuals was counted and used to determine the median effective concentration (48-h EC_{50}). In this study, individuals that were not able to swim within 15 s after gentle agitation of the test vessel were considered to be immobilized.

In chronic toxicity assays, the following nominal concentration ranges were tested: 125 to 8000 and 12.5 to 800 μg diuron L^{-1} dosed as Diuron Nortox® 500 SC and 0.09 to 0.96 and 0.09 to 0.96 μg carbofuran L^{-1} dosed as Furadan® 350 SC. Chronic toxicity tests were conducted using ten replicates, each containing one neonate (6–24 h old) in 15 mL of test solution or 15 mL of reconstituted water (control). Six test concentrations besides a control were tested in the chronic tests with both the active ingredient and commercial product of carbofuran. For the commercial product and active ingredient of diuron, seven and eight different test concentrations besides a control, respectively, were evaluated. In the case of active ingredient diuron, a solvent control (0.01% acetone, *v*/*v*) was included in the test. The duration of the tests was 8 days, and the organisms were fed and maintained under the same conditions (temperature, photoperiod, medium) as described above for culture maintenance. The test solutions were renewed every other day after the number of surviving adults and neonates had been recorded.

To account for the variability and hence the reproducibility of toxicity values, ten definitive acute toxicity tests and three chronic toxicity tests were performed for both test compounds (diuron and carbofuran) and dosing form (active ingredient and commercial product). Acute toxicity tests with the reference substance sodium chloride (NaCl) were carried out monthly to evaluate the physiological condition of the organisms. In addition, water quality parameters (pH, temperature, electrical conductivity, hardness, and dissolved oxygen) were measured using the appropriate probes at the start and at the end of the toxicity tests.

Data analysis

The 48-h EC_{50} values with their 95% confidence intervals for the acute toxicity tests were calculated by nonlinear

regression, using the three-parameter logistic curve in the Statistica 7.0 software (StatSoft 2004). For acute toxicity test data, significant differences between active ingredients and commercial products were verified by Student's *t* test. Chronic toxicity test data were analyzed for significant differences for female survival by Fisher's exact test using the program TOXSTAT 3.3 (Gulley et al. 1991). Reproduction data were first checked for normality (chi-squared test) and homogeneity of variances (Bartlett's test). Since data were in compliance with these parametric assumptions, data were subsequently subjected to one-way analysis of variance (ANOVA). A post hoc multiple comparison Dunnett's test was carried out to verify significant differences between treatments and control. These statistical analyses were performed using Statistica 7.0 software (StatSoft 2004). In all statistical tests, the difference was considered significant when $p \leq 0.05$.

Species sensitivity distribution

Species sensitivity distributions (SSDs) were constructed to compare acute EC_{50} and chronic NOEC values obtained from the *C. silvestrii* tests evaluating diuron and carbofuran with their corresponding values for other invertebrate species. Toxicity data for invertebrates were compiled from the US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/), supplemented with data from the open literature and the draft assessment reports of diuron and carbofuran (EC 2004 a, b) for taxa not included in this database (Supplementary Material Tables S2 and S3). Only laboratory toxicity data for aquatic invertebrates that could be confirmed from original publications and that adhered to the selection criteria provided in Table 1 were included in the SSDs. Geometric means were calculated when more than one toxicity value was reported for a given species. Log-normal distributions of threshold values were constructed using the ETX computer program, version 2.0 (Van Vlaardingen et al. 2004). The 5th percentile (hazardous concentration for 5% of the species—HC5) and 50th percentile (hazardous concentration for 50% of the species—HC50) with their confidence limits were calculated with this software based on the methodology described by Aldenberg and Jaworska (2000). Since the model assumes a log-normal distribution of the data, log-normality was tested with the Anderson–Darling test included in the ETX software package, which was evaluated at the 5% significance level.

Results

Abiotic variables of the toxicity tests and chemical analysis

During the acute and chronic toxicity tests, the measured pH of test solutions remained within the range of 7.1 and 7.6 and did not vary by more than 1.0 unit in any given test. The other water quality parameters in all toxicity tests varied within a narrow range, water temperature 24.2–25.8 °C, electrical conductivity 142.7–164.2 μS cm^{-1}, water hardness 40–48 mg $CaCO_3$ L^{-1}, and dissolved oxygen 6.5–7.3 mg L^{-1}. Thus, all tests met the validity criteria set by ABNT (2005).

Analysis of the test solutions in HPLC-DAD showed that the actual exposure concentrations in acute and chronic toxicity tests with diuron and carbofuran differed by less than 10% from the nominal concentrations (Supplementary Material Figs. S1 and S2). Therefore, the toxicity values were calculated based on nominal concentrations, as suggested by ISO 10706 (2000).

Acute and chronic toxicity

At the end of the acute and chronic toxicity tests, the mortality in both control and solvent control (in the case of active ingredient diuron) did not exceed 10%, as recommended by ABNT (2005). The (acute) reference test using NaCl also indicated that the sensitivity of *C. silvestrii* (48-h EC_{50} ranged from 1.00 to 1.32 g L^{-1}) was within the required range (reference range 1.00 to 1.83 g L^{-1}; Casali-Pereira et al. 2015).

In the acute toxicity tests, 48-h EC_{50} values for diuron and carbofuran both in active ingredient and commercial formulation forms were calculated separately for each of the ten tests performed. The results are presented as box plots in Fig. 1. Toxicity of active ingredients was significantly different from that of corresponding commercial formulations ($p < 0.05$; Student's *t* test). In the case of diuron, the commercial formulation was 7.2 times more toxic than its active ingredient, whereas for carbofuran, the commercial formulation was 1.5 times less toxic than its active ingredient. The results also indicate that the insecticide carbofuran was more toxic than the herbicide diuron to *C. silvestrii*.

In the chronic toxicity tests, the survival percentage of *C. silvestrii* females after 8 days of exposure to 8000 μg L^{-1} diuron was significantly lower than in controls (Fig. 2a). In

Table 1 Selection criteria for acute and chronic toxicity test data

	Acute toxicity tests	Chronic toxicity tests
Endpoint	EC50, LC50	NOEC, EC10
Test parameters	Mortality, immobilization	Growth, feeding, reproduction, development, mortality or immobilization
Test duration (days)	2–4	>6

Adapted from Van den Brink et al. (2006) and Brock and Van Wijngaarden (2012)

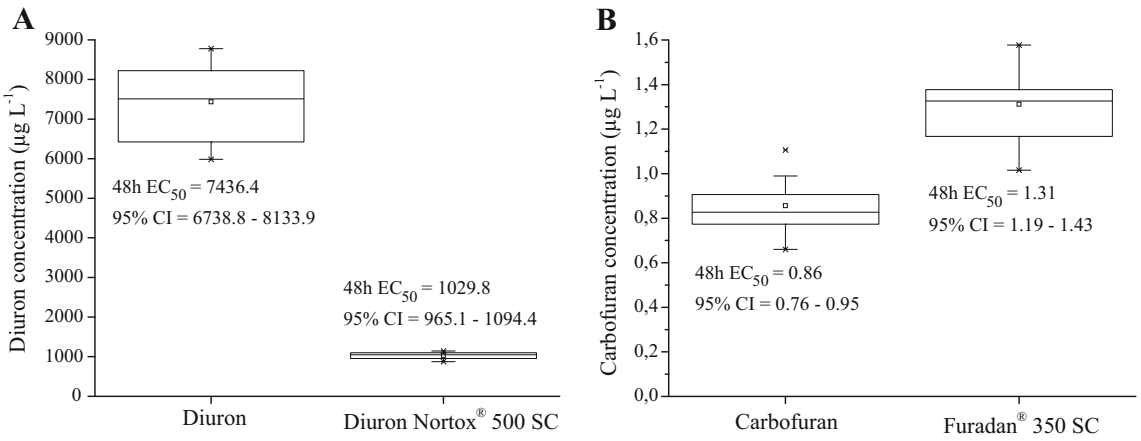

Fig. 1 Box plots representing the 4-h EC_{50} mean values and their 95% confidence intervals for diuron (**a**) and carbofuran (**b**) as calculated for *Ceriodaphnia silvestrii* in the tests with the active ingredient and commercial product. Ten acute toxicity tests were carried out for each compound

experiments with commercial diuron, none of the concentrations tested showed significant differences over controls with respect to survival (Fig. 2b). Regarding survival, the 8-day NOEC was 6000 µg L^{-1} for diuron and 800 µg L^{-1} for commercial diuron, while the 8-day lowest observed effect concentration (LOEC) was 8000 and >800 µg L^{-1} for active ingredient and commercial formulation, respectively. For carbofuran, both in active ingredient and commercial product forms, the female survival percentage at the highest concentration tested (i.e., 0.96 µg a.i. L^{-1}) was significantly lower than in controls (Fig. 2c, d). For survival, the 8-day NOEC and LOEC values for carbofuran (both active ingredient and

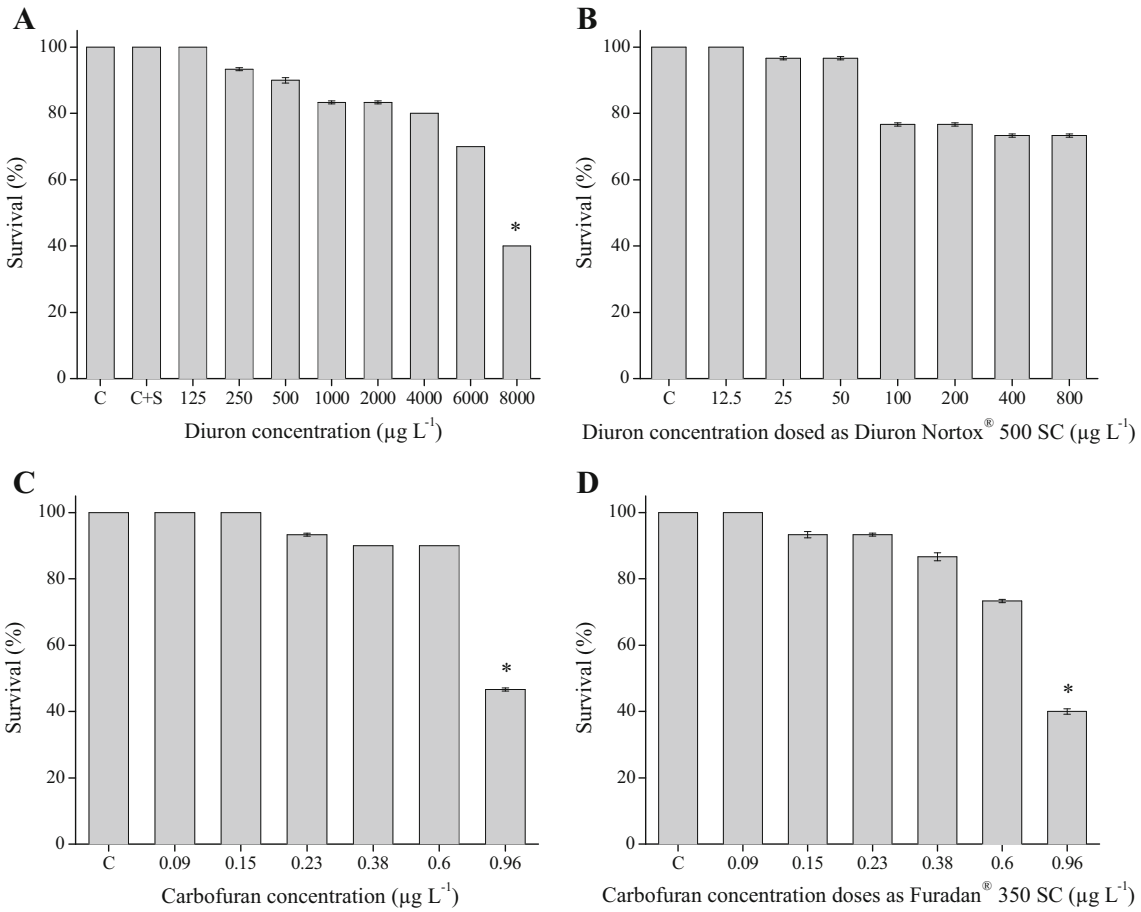

Fig. 2 Survival percentage of *C. silvestrii* females (mean ± SD) after 8 days of exposure to different concentrations of diuron (**a**), diuron dosed as Diuron Nortox® 500 SC (**b**), carbofuran (**c**), and carbofuran dosed as Furadan® 350 SC (**d**) in chronic toxicity tests. Three chronic toxicity tests were carried out for each compound. The *asterisk* indicates the value significantly different from control ($p \leq 0.05$, Fisher's exact test)

commercial formulations) were 0.60 and 0.96 μg L^{-1}, respectively.

The data obtained in chronic toxicity tests show that diuron and carbofuran decreased female *C. silvestrii* fertility at the highest concentrations tested. However, stimulating effects on fertility at low concentrations of diuron were also observed (Fig. 3); diuron caused a significant increase in *C. silvestrii* fertility exposed to concentrations of 250 to 2000 μg a.i. L^{-1} of diuron in its active ingredient form (Fig. 3a) and of 25 to 200 μg a.i. L^{-1} in its commercial form (Fig. 3b). However, a significant decrease in fertility was observed in test organisms exposed to concentrations of 6000 and 8000 μg a.i. L^{-1} of diuron and 400 and 800 μg a.i. L^{-1} when applied as commercial product. All these results were evidenced by Dunnett's test (ANOVA—diuron $F_{9,75} = 47.3$, $p < 0.05$; commercial diuron $F_{7,59} = 51.4$, $p < 0.05$). Regarding fertility, the 8-day NOEC was 125 μg L^{-1} for diuron and 12.5 μg L^{-1} for commercial diuron, while the 8-day LOEC was 250 and 25 μg L^{-1} for active ingredient and commercial formulation, respectively. For carbofuran, both the active ingredient (Fig. 3c) and the commercial product tested in the present study (Fig. 3d) caused a significant decrease in *C. silvestrii* fertility exposed to concentrations of 0.38 μg a.i. L^{-1} and higher, as evidenced by the Dunnett's test (ANOVA—carbofuran $F_{6,56} = 20.2$, $p < 0.001$; commercial carbofuran $F_{6,56} = 40.5$, $p < 0.001$). For fertility, the 8-day NOEC and LOEC values for carbofuran (both active ingredient and commercial formulation) were 0.23 and 0.38 μg L^{-1}, respectively.

SSD

The HC5 and HC50 values with their corresponding 95% confidence intervals, obtained from the SSDs constructed from the acute and chronic toxicity data of diuron and carbufuran, are presented in Supplementary Material Table S4. Due to low data availability, the 95% confidence intervals of especially the HC_5 values in the chronic SSDs are rather large, although the goodness of fit was accepted by the Anderson–Darling test at the 5% significance level for all SSD curves.

By analyzing the SSD curves constructed for diuron (Fig. 4) and carbofuran (Fig. 5), the neotropical cladoceran *C. silvestrii* appears to be among the most sensitive

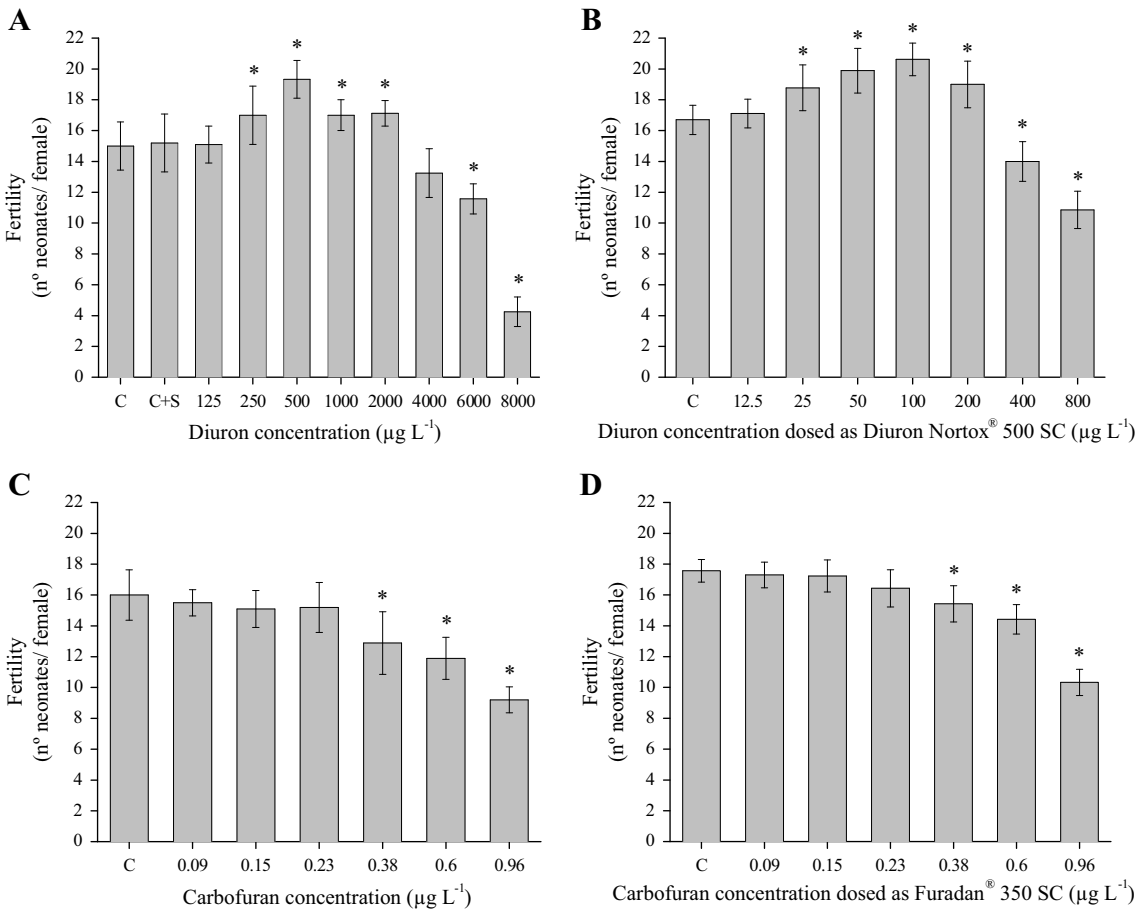

Fig. 3 Fertility of *Ceriodaphnia silvestrii* (mean ± SD number of neonates per female) after exposure 8 days to different concentrations of diuron (**a**), diuron dosed as Diuron Nortox® 500 SC (**b**), carbofuran (**c**), and carbofuran dosed as Furadan® 350 SC (**d**) in chronic toxicity tests. Three chronic toxicity tests were carried out for each compound. The *asterisk* indicates the value significantly different from control ($p \leq 0.05$, Dunnett's test)

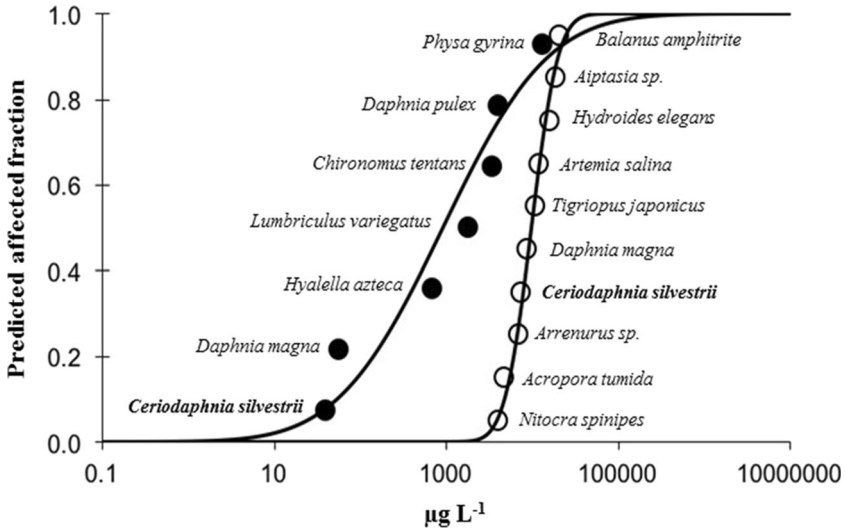

Fig. 4 Species sensitivity distribution (SSD) constructed based on EC_{50} (*open circles*) and NOEC/EC_{10} (*closed circles*) values for diuron obtained in this study for *Ceriodaphnia silvestrii* (in *bold*) and from literature for other invertebrates

invertebrates for which toxicity data were available, especially for carbofuran. Regarding the acute toxicity of diuron, *C. silvestrii* was more sensitive than the temperate standard test species *D. magna* (Cladocera), *Tigriopus japonicus* (Copepoda), *Artemia salina* (Anostraca), *Hydroides elegans* (Polychaeta), *Aiptasia* sp. (Cnidaria), and the barnacle *Balanus amphitrite*. Regarding the SSD curve constructed on chronic (NOEC and EC_{10}) toxicity values, *C. silvestrii* was more sensitive to diuron than all other species represented in the SSD including the standard temperate test species *D. magna*, *Hyalella azteca*, and *Chironomus tentans* (Fig. 4).

The SSD curve for carbofuran (Fig. 5) shows that *C. silvestrii* was the most sensitive invertebrate species included in the species assemblage. Subsequently, *C. silvestrii* was more sensitive to short-term carbofuran exposure than species commonly used in ecotoxicological studies, such as the cladocerans *C. dubia*, *D. magna*, and *Daphnia pulex*; the amphipods *Gammarus fasciatus* and *G. pulex*; the chironomid *Chironomus riparius*; and the rotifer *Brachionus calyciflorus*. Similarly, *C. silvestrii* was more sensitive to chronic carbofuran exposure than the standard test species *Americamysis bahia*, *C. dubia*, and *D. magna*.

Discussion

Acute toxicity of active ingredients and commercial products to *C. silvestrii*

The 48-h EC_{50} mean values calculated for the two active ingredients indicated that carbofuran (48-h EC_{50} = 0.86 µg L^{-1}) was almost four orders of magnitude more toxic to *C. silvestrii* than was diuron (48-h EC_{50} = 7436.4 µg L^{-1}). Given their insecticidal and herbicidal types of action, respectively, this was indeed in line with what was anticipated a priori (e.g.,

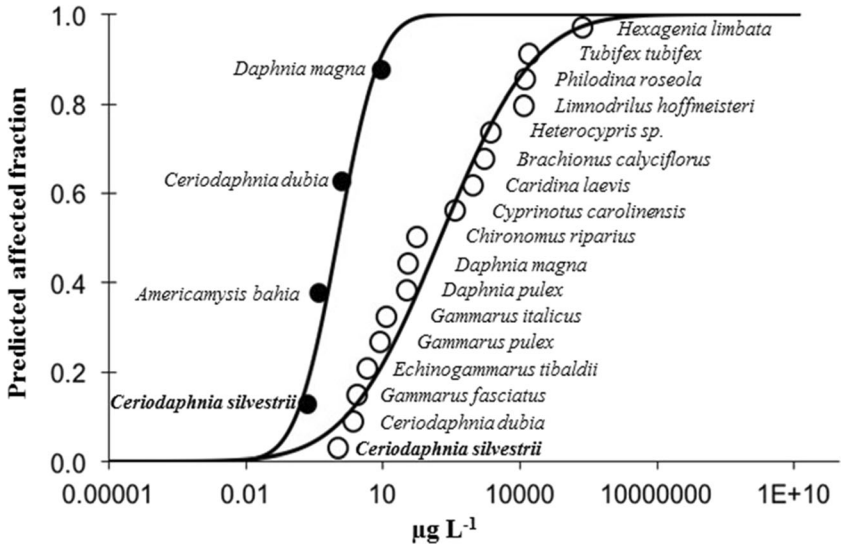

Fig. 5 Species sensitivity distribution (SSD) constructed based on EC_{50} (*open circles*) and NOEC/EC_{10} (*closed circles*) values for carbofuran obtained in this study for *Ceriodaphnia silvestrii* (in *bold*) and from literature for other invertebrates

Maltby et al. 2005). Diuron is a photosynthesis inhibitor, so its main activity is on autotrophic organisms (algae and macrophytes; Van den Brink et al. 2006). Although it has been recognized that diuron may cause toxic effects on heterotrophic nontarget organisms through different modes of action (e.g., AChE inhibition, endocrine disruption activity), a specific mode of action of diuron in cladocerans is still unclear.

In the present study, the toxicity of the active ingredients diuron and carbofuran to *C. silvestrii* was significantly different than that of their commercial products (Diuron Nortox® 500 SC and Furadan® 350 SC, respectively; Fig. 1). Commercial pesticide formulations contain one or more active ingredients aimed at combating the target organism for which the pesticide product was developed. In addition, formulations contain inert compounds that act as solvents, emulsifiers, surfactants, etc. (Cox and Surgan 2006). These inert ingredients may influence the toxicity of the pesticide formulation directly though intrinsic toxicity or indirectly by interacting with the toxicity of the active ingredient. In the majority of cases where differences in toxicity of active ingredients and their formulated products are encountered, the toxicity of the formulated product is greater than that of the active ingredient (e.g., Pereira et al. 2009; Beggel et al. 2010; Mullin 2015), as was the case in the present study for diuron and its formulated product Diuron Nortox® 500 SC (Fig. 1a). In line with this, Kroon et al. (2015) reported that estrogenic biomarkers in juvenile barramundi (*Lates calcarifer*) increased following exposure to a commercial mixture of diuron (Diurex® WG) but not to the analytical grade diuron, suggesting an estrogenic response to the additives.

Although less common, the toxicity of an active ingredient can also be greater than that of a commercial formulation, as was noted for carbofuran and commercial carbofuran in the present study. In such cases, the inert ingredients in the formulation apparently have an antagonistic action on the toxicity of the active ingredient (e.g., Guilherme et al. 2012). Interestingly, previous experiments conducted at our laboratory with the rotifer *Philodina roseola* also demonstrated a greater toxicity of analytical grade carbofuran as compared to Furadan® 350 SC (Moreira et al. 2015).

Sensitivity of *C. silvestrii* to chronic carbofuran and diuron exposure

The chronic 8-day tests with carbofuran and its commercial product revealed a significant decrease in survival at the highest concentration tested (0.96 μg a.i. L^{-1}; Fig. 2c, d). The 40–50% survival at this test concentration are in accordance with the 48-h EC_{50} of 0.86 μg a.i. L^{-1} discussed in the previous section. This absence of an increase in toxicity with exposure duration may be explained by the fact that carbofuran is a reversible AChE inhibitor (IRAC 2016). Subsequently, female survivors might adapt to prolonged exposure by spontaneous reactivation of inhibited AChE and/or of new synthesis of this enzyme (Baldwin et al. 2009). Furthermore, unlike in the acute tests, animals in the chronic tests were supplied with food. Both forms of carbofuran exerted significant effects on the fertility of *C. silvestrii* females from a concentration of 0.38 μg a.i. L^{-1} onwards, with severity of effects increasing with increasing test concentration (Fig. 3c, d). Several other studies have reported effects of low pesticide concentrations on cladoceran reproduction, decreasing the number of neonates and increasing embryo abnormalities (e.g., Palma et al. 2009; Freitas and Rocha 2012), including *C. silvestrii* (e.g., abamectin; Casali-Pereira et al. 2015).

Interestingly, both forms of diuron caused increased female fertility at intermediate test concentrations and decreased fertility was only observed at higher treatment levels (Fig. 3a, b) at concentrations that did not cause significant effects on survival (Fig. 2a, b). The stimulatory response at low concentrations in this so-called hormesis process is either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis (Calabrese and Baldwin 2002). Hormesis has been demonstrated in several aquatic species exposed to a variety of chemical compounds including invertebrates exposed to sublethal concentrations of pesticides (e.g., Calabrese and Baldwin 2003; Zalizniak and Nugegoda 2006; Cedergreen et al. 2007; Li and Tan 2011; Moreira et al. 2015). Li and Tan (2011), for example, demonstrated hormetic effects on cholinesterase activity in *D. magna* after chronic exposure to intermediate test concentrations of triazophos and chlorpyrifos. The evaluation of hormesis has been discussed to be especially relevant for pesticides since they are generally present in low concentrations in the environment (Konstantinou et al. 2006; Tyne et al. 2015). Several studies have suggested different mechanisms to explain hormesis, which are generally related with an increase in energy acquisition or changes in energy allocation (e.g., Forbes 2000; Calabrese and Baldwin 2002; Jager et al. 2013). Although increased female fertility at intermediate pesticide concentrations could be interpreted as a positive response, this could have severe consequences for population survival and fitness through a combination of disproportionate energy allocation to reproduction and stress caused by the pesticide exposure. In addition, it may result in direct and latent effects on offspring. Zalizniak and Nugegoda (2006), for example, demonstrated that sublethal chlorpyrifos concentrations caused hormetic effects on first and second generations of exposed *Daphnia carinata* adults but that third-generation offspring was more sensitive to this insecticide.

Comparison of the sensitivity to pesticides between *C. silvestrii* and other invertebrates

The SSD curves in Figs. 4 and 5 allowed a comparison of the relative acute and chronic sensitivity of *C. silvestrii* to diuron

and carbofuran, respectively, with that of other invertebrate species reported in the open literature. From these figures, it appeared that *C. silvestrii* was more sensitive to both compounds than standard invertebrate test species commonly used in temperate regions, such as *C. dubia*, *D. magna*, and *D. pulex*. Based on their geometric mean EC_{50} ($gmEC_{50}$) values, *C. silvestrii* was approximately 3 and over 40 times more sensitive to acute carbofuran exposure than *C. dubia* and *D. magna*, respectively. *C. silvestrii* also appeared to be approximately 35 times more sensitive to chronic carbofuran exposure (8-day NOEC, fertility = 0.23 μg a.i. L^{-1}) than *D. magna* (21-day NOEC, number of surviving neonates = 8 μg a.i. L^{-1}; EC 2004b). Besides the acute diuron exposure, for which only the copepod *Nitocra spinipes*, the cnidarian *Acropora tumida*, and the water mite *Arrenurus* sp. appeared (less than two times) more sensitive, *C. silvestrii* was the most sensitive invertebrate to acute and chronic carbofuran and chronic diuron exposure (Figs. 4 and 5).

Previous studies also encountered greater sensitivities of indigenous tropical species as compared with their temperate counterparts (e.g., Do Hong et al. 2004; Freitas and Rocha 2012; Moreira et al. 2014). Do Hong et al. (2004), for example, demonstrated that *C. cornuta* was more sensitive to methyl-parathion and diazinon as compared to *D. magna*. It should be noted, however, that there does not appear to be a consistent greater sensitivity (or tolerance) of tropical species as compared to temperate species (e.g., Daam and Van den Brink 2010; Rico and Van den Brink 2011). In addition, experimental factors related with species (e.g., size and age) and experimental conditions (e.g., temperature, photoperiod, and water quality parameters such as hardness and pH) may influence sensitivity comparisons (e.g., Kwok et al. 2007; Freitas and Rocha 2012; Moreira et al. 2014). However, the use of indigenous species in tropical effect assessments is recommended for several reasons, including (i) a more ecologically relevant assessment of the true sensitivity and subsequently the potential risk of tropical freshwater life, (ii) direct availability and hence less logistic constraints, and (iii) to avoid introducing temperate exotics in tropical ecosystems (e.g., Daam and Van den Brink 2010; Freitas and Rocha 2011; Rico and Van den Brink 2011).

Conclusions on the potential risks of diuron and carbofuran

In Brazil, both carbofuran and diuron have been detected in several edge-of-field water bodies. Maximum reported carbofuran concentrations range from 0.1 to 68.8 μg L^{-1} (e.g., Carbo et al. 2008; Caldas et al. 2011; Ribeiro et al. 2013; Loro et al. 2015). At these concentrations, ecological acute and chronic effects on *C. silvestrii* are likely to occur when considering the toxicity values derived in the present study (48-h EC_{50} = 0.86 μg L^{-1}; 8-day LOEC, fertility = 0.38 μg L^{-1}). The HC_5 obtained from the acute SSD (0.35 μg L^{-1}; Table S4) also indicates that several other invertebrates may be expected to be adversely affected at these concentrations. A potentially affected fraction of 40% is indicated by the SSD at a concentration of 68.8 μg L^{-1}.

For diuron, maximum reported concentrations range from 0.9 to 408 μg L^{-1} (e.g., Paschoalato et al. 2008; Dores et al. 2009; Dantas et al. 2011; Britto et al. 2012). No direct acute effects on *C. silvestrii* are to be expected at these concentrations based on the toxicity values obtained in the present study (48-h EC_{50} = 7436.4 μg L^{-1}). Based on the acute SSD, only a small percentage of other invertebrates may also be expected to be at risk at these concentrations (HC_5 = 19.7 μg L^{-1}; Table S4). However, hormetic effects on fertility may occur at these exposure levels and cause negative side effects as discussed in previous sections (8-day LOEC, fertility = 250 and 25 μg L^{-1} for the active ingredient and formulated product, respectively). This may be aggravated indirectly through the occurrence of an additional stressor caused by a reduction in food availability in the form of edible algal biomass. Brock et al. (2000), for example, reported $gmEC_{50}$ and gmNOEC for a range of algal exposure to diuron for 72–96 h of 15–51 μg L^{-1} and 2.3–7 μg L^{-1}, respectively. Given that also macrophytes may be expected to be impacted at the reported diuron concentrations (e.g., 7-day NOEC *Lemna major* and *L. perpusila* = 2.3 μg L^{-1}; Brock et al. 2000), effects on ecosystem structure including indirectly on cladocerans like *C. silvestrii* are hence likely to occur at diuron concentrations encountered in Brazilian edge-of-field water bodies. In addition, it should be noted that the laboratory tests were conducted under controlled conditions, whereas organisms in the field may be subjected to several additional stressors (e.g., limited abiotic conditions, additional pollutants, competition for resources, and predation) that may increase their sensitivity.

The results of this study also show that the sole evaluation of active ingredients or commercial formulations in risk assessments is insufficient. As observed for diuron and carbofuran, the commercial formulations may cause a higher or a lower toxicity to *C. silvestrii* than their active ingredients. Therefore, risk assessments in the tropics should be conducted for both commercial formulations and the active ingredients they contain, as is also already requested in, e.g., the EU (EFSA 2013).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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