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Effects of diuron and carbofuran pesticides in their pure and commercial forms on *Paramecium caudatum*: The use of protozoan in ecotoxicology☆

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ABSTRACT

Toxic effects of diuron and carbofuran on *Paramecium caudatum* were evaluated. Acute and chronic tests were conducted with diuron and carbofuran active ingredients and their commercial formulations, Diuron Nortox® 500 SC and Furadan® 350 SC, respectively. The sensitivity range of *P. caudatum* to reference substance sodium chloride was established. A preliminary risk assessment of diuron and carbofuran for Brazilian water bodies was performed. The tests indicated that toxicity of pure diuron and its commercial formulation was similar, while the commercial product carbofuran was more toxic than its pure form. In acute tests, readings were carried out at 2, 3, 4 and 6 h and showed an increase of mortality with increasing exposure time. The sensitivity of *P. caudatum* to NaCl ranged from 3.31 to 4.44 g L^{-1}, averaging 3.88 g L^{-1}. For diuron, the 6 h LC_{50} was 64.6 ± 3.3 mg L^{-1} for its pure form and 62.4 ± 2.5 mg L^{-1} for its commercial formulation. Carbofuran active ingredient was less toxic than that of diuron, presenting a 6 h LC_{50} of 142.0 ± 2.4 mg L^{-1} for its pure form and 70.4 ± 2.2 mg L^{-1} for its commercial product. Chronic tests showed that these pesticides cause significant decrease on population growth, generation number and biomass of *P. caudatum*. The 24 h IC_{50} was 7.10 ± 0.58 mg L^{-1} for pure diuron, 6.78 ± 0.92 mg L^{-1} for commercial diuron, 22.95 ± 3.57 mg L^{-1} for pure carbofuran and 4.98 ± 0.62 mg L^{-1} for commercial carbofuran. Preliminary risk assessment indicated that diuron and carbofuran present potential ecological risks for Brazilian water bodies. *P. caudatum* was a suitable and sensitive test organism to evaluate diuron and carbofuran toxicity to freshwater protozooplankton and, taking into account the relevant role of protozoans in aquatic environments, we strongly recommend its inclusion in ecotoxicological studies.

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1. Introduction

Pesticides are deemed necessary to boost farm productivity, but its indiscriminate use may cause severe problems due to environmental contamination which can lead to large losses of biodiversity (Carvalho, 2006) and public health problems (Daam and Van den Brink, 2010). One of the main problems with the use of these compounds is that as they are not fully selective, they can cause toxic effects in non-target organisms, representing a threat to terrestrial and aquatic ecosystems normal functioning (Schreinemachers and Tipraqsa, 2012). When applied to the soil and/or plants, and depending on the persistence of the chemical in the environment, pesticides are subjected to a series of biotic and abiotic processes that can involve the degradation and/or transport through drift, leaching and runoff to different environmental matrices (e.g. water, sediment, soil) (Wilson and Foos, 2006). Thus, the considerable contamination risk of the water in the environment is quite evident, especially when an agricultural ecosystem and water bodies are in close proximity (Abhilash and Singh, 2009).

Geoclimatic conditions of each ecosystem directly influence the toxic effects of the pesticides to non-target organisms. Thus, environmental risk assessments of these substances in tropical

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conditions are essentially recommended (Daam and Van den Brink, 2010), considering that in the tropics, due to the relatively high mobility of pesticides in soil and frequent torrential rains in the summer, many defensives applied to crops are leached and, their residues may be found in surface and groundwater (Pinheiro et al., 2010; Wightwick and Allinson, 2007). In addition, factors such as soil quality, which affects the mobility of the compounds, and higher temperatures, which increase the solubility of the products in water, and the absorption by non-target organisms, enhance environmental risks (Sanches-Bayo and Hyne, 2011).

Brazil is the world's largest consumer of pesticides, followed by USA (Pelaez et al., 2013). Among the pesticides, diuron and carbofuran occupied the 13th and 26th position in the sales ranking being that 6100.96 tons active ingredient and 1739.81 tons active ingredient, respectively, were sold in 2013 (IBAMA, 2015). The state of São Paulo is the region with the largest use of diuron and carbofuran, representing, respectively, 41% and 62% of total sales, being mainly used in sugarcane plantations. However, in several regions of Brazil contamination of surface and groundwater by these pesticides have already been reported. Maximum concentrations detected for diuron were in the range of 0.9–408 μg L^{-1} (Britto et al., 2012; Dantas et al., 2011; Dores et al., 2009; Paschoalato et al., 2008) and for carbofuran were in the range of 0.1–68.8 μg L^{-1} (Caldas et al., 2011; Carbo et al., 2008; Loro et al., 2015; Ribeiro et al., 2013).

Diuron is an herbicide belonging to the phenylamide family and the subclass of phenylurea. This compound inhibits photosynthesis by preventing oxygen production and blocking the electron transfer at the level of photosystem II in photosynthetic microorganisms and plants (Giacomazzi and Cochet, 2004). It is extensively used on many agricultural crops such as sugarcane, citrus, cotton, coffee, and others and its dispersion can lead to the pollution of the aquatic environment mainly by soil leaching as indicated by its Groundwater Ubiquity Score (GUS) index of 2.58 (Cerdeira et al., 2015; Gooddy et al., 2002). Due to its high persistence (one month to one year), diuron can be found in many matrices such as soil, sediment and water (Field et al., 2003). Diuron is known to be slightly toxic to mammals and birds, moderately toxic to fish and slightly toxic to aquatic invertebrates (Giacomazzi and Cochet, 2004).

Carbofuran is an insecticide, acaricide and nematicide known to bind to the acetylcholinesterase enzyme, inhibiting its action on the acetylcholine (Pessoa et al., 2011). This pesticide carbamate is used in increasing amounts on fields of rice, cotton, coffee, sugarcane, beans and corn (Ehler, 2004). Aquatic environments are particularly prone to carbofuran contamination as indicated by its GUS of 4.52, characterizing a relatively high risk of being transported from the soil of application areas to adjacent water bodies after rain events (Carbo et al., 2008; Ribeiro et al., 2013). Carbofuran is considered highly toxic to birds, bees and aquatic animals, such as fishes (Collective SPA, 2002; Ibrahim and Harabawy, 2014).

An in-depth appraisal of the consequences of the presence of carbofuran and diuron for aquatic life would require, evidently, a more systematic investigation, involving a variety of test organisms. Among the non-target organisms, ciliated protozoa are important components of the aquatic ecosystem and play a crucial role in the food webs functioning (Madoni, 2006; Rehman et al., 2008). They act as a link between bacterial production and secondary producers (Fenchel, 1987) and perform important functions such as: remineralization process increase (Sherr and Sherr, 1984), bacterial density control (Mansano et al., 2014) and changes in morphological and taxonomic composition of bacterial communities by predation (Corno et al., 2008). Furthermore, protozoans are prey of higher organisms and, as a consequence, contaminants can be potentially transferred along food chains and affect organisms at higher trophic levels (Gerhardt et al., 2010; Mortuza et al., 2005). Thus, effects of toxicants on protist components of microbial communities may alter the trophic chain and significantly affect the aquatic ecosystems balance (Trielli et al., 2007).

Adding to the ecological attributes, ciliated protozoans also have the advantages of short life cycles, cosmopolitan distribution, structural simplicity, high degree of reproducibility and quick responses to environmental disturbances in an integrated and continuous manner (Tan et al., 2010). Moreover, several species of protozoans can be cultured in laboratory under conditions very similar to those in nature, making their biological responses more reliable (Delmonte Conrrado et al., 2006).

Studies on interactions between ciliates and environmental pollutants are scarce compared with those of other microorganisms (e.g., bacteria and algae), despite the fact that ciliates are easy to keep in culture and considered to be reliable biological models and hence good candidates for use in bioassays (Delmonte Conrrado et al., 2006; Gomiero et al., 2013). Tests performed with the eukaryotic cell of *Tetrahymena pyriformis* showed significantly higher sensitivity to diverse xenobiotics, e.g. heavy metals and insecticides, compared to bacterial tests such as the Microtox® test (Bogaerts et al., 2001; Bonnet et al., 2007).

The general aim of this study was to assess the acute and chronic toxic effects of the pesticides diuron and carbofuran, employing the ciliated protozoan *Paramecium caudatum*. The specific aims were: (I) To determine the sensitivity range of this protozoan to a reference substance, with a view to its eventual routine use in laboratory tests; (II) To determine the LC_{50} (2, 3, 4 and 6 h) of the pesticides carbofuran and diuron testing pure active ingredient and commercial product; (III) To assess the chronic effects (24 h) of the carbofuran and diuron (in pure and commercial form) on population growth, generation number and biomass variables; (IV) To perform a preliminary risk assessment of the pesticides diuron and carbofuran for Brazilian water bodies.

2. Materials and methods

2.1. Stock maintenance and culture of ciliated protozoan

Paramecium caudatum (Protozoa, Ciliophora) was initially isolated from Monjolinho Reservoir, São Carlos, SP, Brazil and kept in stock cultures for more than 5 years in the Culture Collection of the Aquatic Microorganisms Ecology Laboratory at the Federal University of São Carlos.

Ciliates were cultured in tubes containing: inclined agar (2%), a sterile rice grain in the husk and Minalba® mineral water supplemented with *Enterobacter aerogenes* bacteria at final concentration of 10^6 cells mL^{-1} (Madoni and Romeo, 2006; Rao et al., 2007; Wickham and Gugenberger, 2008). *E. aerogenes* bacteria that served as food for the protozoans were grown in Nutrient Agar medium (pH 6.8 ± 0.2) at constant temperature of 35 ± 2 °C for 24 h (Difco and BBL, 2009). The chemical characteristics of the mineral water used as culture medium were: bicarbonates 97.51 mg L^{-1}, calcium 16.11 mg L^{-1}, magnesium 9.08 mg L^{-1}, potassium 1.02 mg L^{-1}, sodium 0.92 mg L^{-1}, nitrates 0.70 mg L^{-1}, sulfates 0.13 mg L^{-1}, chlorides 0.11 mg L^{-1}, fluorides 0.05 mg L^{-1}, barium 0.02 mg L^{-1}, strontium 0.02 mg L^{-1}, pH 7.89.

The *P. caudatum* stock cultures were kept in incubator at controlled temperature of 25 ± 1 °C; photoperiod of 12 h light: 12 h dark and pH 7.5–8.0 (adapted from Rao et al., 2007). Ciliates were pricked out into new culture medium, at least every two weeks for their maintenance. New cultures were initiated by seeding 10 mL of the medium with 50 organisms of *P. caudatum* from a stationary phase culture. Only individuals from populations at logarithmic growth phase (stock culture < 72 h based on a preliminary growth

experiment) were used for tests.

2.2. Test substances and solutions

The five chemical products tested in the toxicity assays were: sodium chloride (NaCl), from Lafan Química Fina Ltda (Brazil), diuron (N-(3,4-dichlorophenyl)-N,N-dimethyl-urea) from Sigma–Aldrich, Diuron Nortox® 500 SC, from Nortox S/A (Brazil), carbofuran (2.3-dihydro-2.2-dimethyl-7- benzofuranyl N-methylcarbamate) from Sigma–Aldrich, and Furadan® 350 SC, from FMC (Brazil). The first compound was chosen because it is a reference toxic substance suitable for routine use in sensitivity testing (Environmental Canada, 1990), and was used here to establish the sensitivity range of *P. caudatum*. The following compounds were the pesticides used in the acute and chronic toxicity tests, which were conducted using both pure active ingredient and commercial formulation.

The chemicals sodium chloride, diuron and carbofuran had a high purity (>99%, >98% and >98%, respectively). The purity levels of the commercial formulation Diuron Nortox® 500 SC is 50% m/v of active ingredient (69,4% m/v of inert ingredients) and Furadan® 350 SC is 35% m/v of active ingredient (65% m/v of inert ingredients). The stock solutions of sodium chloride (100 g L^{-1}), diuron dosed as Diuron Nortox® 500 SC (1000 mg L^{-1}), carbofuran (300 mg L^{-1}) and carbofuran dosed as Furadan® 350 SC (1000 mg L^{-1}) were prepared by dilution of a specific amount of each compound in distilled water just before the tests, with the exception of the pure 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). In turn, the nominal concentrations of each compound tested were obtained by dilution of the stock solution in culture medium (mineral water Minalba®).

To confirm the nominal concentrations used in the tests, stock solutions and test concentrations were analyzed in an Agilent Technologies series 1200 high-performance liquid chromatography (HPLC) (Waldbronn, Germany), equipped with a diode array detector (DAD). The chromatographic analysis conditions were: Agilent Zorbax ODS C18 column (250 mm × 4.6 mm × 5 μm) (Agilent Technologies, USA) and oven temperature of 25 °C. The isocratic mobile phase utilized was acetonitrile and Milli-Q water (70:30, v/v) for 6 min, followed by post time of 2 min, at an injection volume of 20 μL and a flow rate of 1.0 mL min^{-1}. Analyses were carried out in three replicates. HPLC-grade organic solvent (acetonitrile) was obtained from Merck (Germany). Based on absorbance signals observed in the DAD spectrum of the standard solutions and from the literature (Cappelini et al., 2012; Liyanage et al., 2006; Qun and Rohrer, 2012; Rocha et al., 2013), diuron and carbofuran were detected and quantified at 254 nm and 280 nm, respectively. The retention times found for carbofuran and diuron were 3.620 and 4.271 min, respectively. The precision in terms of repeatability, expressed as relative standard deviation (RSD), was 0.76% for diuron and 1.38% for carbofuran.

2.3. Acute and chronic toxicity tests

The acute toxicity test was carried out based on experimental design developed by Moreira et al., 2015 for the rotifer *Philodina roseola*, with necessary changes for the toxicological study using protozoan (exposure period and test solution volume). For acute toxicity testing 5 concentrations were used plus a control, whereas for the chronic toxicity tests 6 concentrations plus a control were established. For both, the acute and chronic toxicity tests, a minimum of 5 concentrations plus a control (ASTM, 2004; OECD, 2008) are recommended, although it is not required to limit the treatments to this number. After a series of preliminary tests, the range of concentrations for each compound was established and acute toxicity tests were carried out at the following nominal concentration ranges: 3–5.5 g L^{-1} of sodium chloride, 40–140 mg L^{-1} of diuron and 14–224 mg L^{-1} of diuron dosed as Diuron Nortox® 500 SC, 120–240 mg L^{-1} of carbofuran and 60–180 mg L^{-1} of carbofuran dosed as Furadan® 350 SC.

The sodium chloride acute toxicity tests were repeated 20 times, at intervals varying between 30 and 60 days, to establish the sensitivity range (or control chart) of *P. caudatum* to this compound. For diuron and carbofuran using both the pure active ingredient and the commercial formulation, the acute toxicity tests were repeated 5 times, in order to determine the acute toxicity of these products to *P. caudatum*.

Acute toxicity assays were conducted in watch-glasses (80 mm diameter) kept individually inside glass Petri dishes (110 mm diameter), for a maximum exposure period of 6 h. This exposure time was chosen considering the *P. caudatum* replication time obtained under good health conditions (9.2 h mean time). The exposure time should be less than the protozoan replication time in order to evaluate the toxicity at the individual level (endpoint mortality). Ten ciliates were transferred from the stock culture, in the logarithmic phase, to each watch-glass by means of a glass micropipette (capillary), under a Leica MZ6 stereo microscope, at 50 × magnification. To prevent dilution of the test solution, the culture medium carried over with the protozoan was drained with the capillary pipette from the watch-glass, and then it was added the test solution.

The control solution for all tests consisted of mineral water alone. In the case of pure diuron, an additional control (mineral water plus acetone) was necessary to determine the effect of the solvent on the survival of the organisms. The amount of solvent added in the tests was always equal to 0.5% (v/v), which did not cause any toxic effects in the preliminary tests (data not shown). For each test-concentration and control, four replicates were carried out, containing 1 mL of test solution and 10 organisms in each replicate. The experiments were maintained at 25 ± 1 °C, without addition of food and in absence of light. The pH, electrical conductivity, temperature and hardness were measured in the test and control solutions at the beginning and end of each test. For each toxicity test, non-toxic plastic cups containing 100 mL of each test solution and the control solution were prepared before the test. The 1 mL aliquots used in each test were taken from these 100 mL volumes and the remaining volume, which was sufficient for the variables to be measured, was placed in the incubator together with the tests and controls.

During the 6 h period, readings were performed on the exposure times of 2, 3, 4 and 6 h and number of dead individuals in the four replicates was counted under the stereo microscope. For each exposure time, the median lethal concentration (LC_{50}) value was calculated. In this study, lysed, deformed or disappeared cells were considered dead individuals.

For chronic toxicity tests we adapted from the methods already described in the literature (e.g. Bonnet et al., 2007; Gomiero et al., 2013; Rao et al., 2007). Three complete chronic toxicity tests were performed with diuron and carbofuran using both the pure active ingredient and the commercial product. The bioassays were carried out at the following nominal concentration ranges: 0.88–28 mg L^{-1} of diuron and of diuron dosed as Diuron Nortox® 500 SC, 1.88–60 mg L^{-1} of carbofuran and 0.47–15 mg L^{-1} of carbofuran dosed as Furadan® 350 SC. These levels were chosen based on acute toxicity preliminary tests.

Chronic toxicity tests had 24 h duration and were performed in test tubes (size 15 × 100 mm) containing 5 mL of test solution and 50 protozoans, coming from a culture at the logarithmic phase. The control consisted of Minalba® mineral water plus *E. aerogenes* bacteria (final concentration of 10^6 cells mL^{-1}) and a sterile rice

grain. The different treatments consisted of culture medium equal to the control, but contaminated with the selected pesticides. An additional control using filtered natural freshwater from Monjolinho Reservoir plus *E. aerogenes* bacteria (final concentration of 10^6 cells mL^{-1}) and a sterile rice grain was performed. Four replicates were carried out for both controls and each concentration of pesticide. The experiments were maintained under the same temperature and photoperiod of stock cultures: 25 ± 1 °C and 12 h light: 12 h dark, respectively.

After 24 h exposure, protozoans were fixed with 0.4% acid lugol and the cells were quantified and measured under optical microscope (100 × magnification) using Sedgwick-Rafter chamber. Protozoan number in each concentration after 24 h was used to determine the concentration of pesticide required for a 50% growth inhibition (IC_{50}). Biovolumes were determined by measuring *P. caudatum* cell dimensions ($n = 30$) and using the appropriate equation for the prolate spheroid geometric shape (Hillebrand et al., 1999). The protozoan biomass was calculated using the cell biovolume converted into carbon by using specific conversion factor for Lugol-fixed ciliates (0.19 pg C μm^{-3}) according to Putt and Stoecker (1989), multiplied by the number of *P. caudatum* individuals. Based on the cell densities, the number of generations (fissions) was estimated by the following formula (Rao et al., 2007): $n = (\text{Log } N_1 - \text{Log } N_0)/\text{Log } 2$, where n is the number of generations; N_1 is the number of cells at 24 h and N_0 is the number of cells at 0 h.

2.4. Data treatment and statistical analysis

The LC_{50} for acute toxicity tests and IC_{50} for growth inhibition tests were calculated by nonlinear regression, using the three-parameter logistic curve in the Statistica 7.0 software (Statsoft, 2004). Given that the use of EC_{10} values to express toxicity in chronic tests has increased over the past years (e.g. Jager, 2012; Landis and Chapman, 2011), EC_{10} or IC_{10} values were also calculated in the chronic tests using three-parameter logistic curve.

The sodium chloride sensitivity range of *P. caudatum* was established by means of a model developed by the USEPA (1985). The control chart consisted of a plot of the 6 h LC_{50} values calculated for each of the 20 assays. The upper and lower limits of the range are shown as two lines which correspond to two standard deviations above and below the mean value, respectively.

Chronic toxicity tests data (growth inhibition, number of replications and biomass) were first checked for normality (with the Chi squared test) and homogeneity of variances (with Bartlett's test), and then subjected to one-way analysis of variance (ANOVA). A post hoc multiple comparisons Dunnett's test was carried out when differences were revealed in data that followed a normal distribution. When the normality test failed, a non-parametric Kruskal–Wallis test was used and multiple comparisons were performed by Dunn's method. In the statistical tests, differences were considered significant when $p \leq 0.05$. All statistical analyzes were performed using Statistica software version 7.0 (Statsoft, 2004).

2.5. Preliminary risk assessment

Preliminary risk assessment of the pesticides diuron and carbofuran was assessed on the basis of the risk quotient (RQ). Risk quotients were calculated from the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC) of these compounds, expressed as the PEC/PNEC ratio (RQ = PEC/PNEC) (Cristale et al., 2013). PEC values for diuron and carbofuran were estimated using the model FOCUS – Forum for the Coordination of Pesticide Fate Models and their Use – Steps 1–2 version 3.2, available in http://focus.jrc.ec.europa.eu/sw/index.html. Input data used for the PEC calculations of diuron and carbofuran in the FOCUS model were obtained from Pesticide Properties Data Base (PPDB). In this study, the selected base crop was sugarcane, to which application rates of Diuron Nortox® 500 SC (diuron) and Furadan® 350 SC (carbofuran) are 3.2 kg a.i. ha^{-1} and 1.75 kg a.i. ha^{-1}, respectively (AGROFIT, 2015). PNEC values were calculated using the following equation: PNEC = LC_{50} or EC_{10}/AF, where LC_{50} is the value obtained from the acute toxicity test, EC_{10} is the value obtained from the chronic toxicity test and AF is the assessment factor. In this study, the AF of 1000 was used for acute toxicity data and the AF of 100 was used for chronic toxicity data (European Commission, 2011; OECD, 2011). For data interpretation, RQ < 1 indicates no significant risk and RQ ≥ 1 indicates a potential risk (Papadakis et al., 2015; Vryzas et al., 2009).

3. Results

3.1. Abiotic variables of the toxicity tests and chemical analysis

During acute and chronic toxicity tests the measured pH of test solutions remained within the range of 7.6 and 8.4 and did not vary by more than 1.0 unit in any given test. The water temperature of the test solutions in all toxicity tests varied between 24 and 25 °C, the electrical conductivity ranged from 151.6 to 157.9 μS cm^{-1} and the water hardness varied between 76 and 80 mg $CaCO_3$ L^{-1}.

After analyzing the test solutions in HPLC-DAD, our results showed that the actual exposure concentrations in acute and chronic toxicity tests of both chemicals (diuron and carbofuran) differed by less than 10% from the nominal concentrations (Supplemental Figs. S1 and S2). Therefore, the results are given in nominal concentrations, as suggested by ISO 10706 (2000).

3.2. Acute toxicity

At the end of the tests, the mortality in both control and solvent control (in the case of pure active ingredient diuron) did not exceed 10%, as recommended by the OECD guidelines. The sensitivity (6 h LC_{50}) of *P. caudatum* to sodium chloride (NaCl) ranged from 3.31 to 4.44 g L^{-1}, averaging 3.88 g L^{-1} (Supplemental Fig. S3). The LC_{50} mean values at 2, 3, 4 and 6 h and their respective 95% confidence intervals, for each of the five compounds analyzed are presented in Table 1. The mortality (%) endpoint for the protozoans exposed to pure and commercial form of diuron and carbofuran at different exposure periods are reported in Fig. 1.

3.3. Chronic toxicity

For all the compounds tested, the variation coefficient of the replicates in the controls and in the treatments of the population growth tests did not exceed 10%, as recommended by the OECD guidelines. Detailed information on the growth of *P. caudatum* is described in a parallel study on life-cycle this species (manuscript in preparation to be published), in which it was observed that the mean replication time is 9.2 h for healthy cultures at 25 °C. Thus, after 24 h, the *P. caudatum* should replicate approximately 2.6 times. This was confirmed in the control groups in the present chronic toxicity tests. Control using mineral water as culture medium and additional control using filtered natural freshwater from Monjolinho Reservoir showed no significant differences ($p = 0.401$) regarding *P. caudatum* population growth.

The mean values of 24 h IC_{10} and 24 h IC_{50} and their respective 95% confidence intervals, for each of the pesticides analyzed are presented in Table 1. Fig. 2 shows the effect of growth inhibition (%) on *P. caudatum* exposed to the selected pesticides. For diuron, both pure active ingredient (Fig. 2A) and its commercial product (Fig. 2B)

Table 1
Values of LC_{50} (2, 3, 4 and 6 h of exposure time) for acute toxicity and 24 h IC_{10} and IC_{50} values for chronic toxicity of five compounds tested and 95% confidence intervals (in parenthesis) obtained for the ciliated protozoan *Paramecium caudatum*.

Compounds tested	Acute tests				Chronic tests	
	2 h LC_{50}	3 h LC_{50}	4 h LC_{50}	6 h LC_{50}	24 h IC_{10}	24 h IC_{50}
Sodium chloride (g L^{-1})	5.05 (4.94–5.15)	4.79 (4.59–5.00)	4.42 (4.22–4.61)	3.88 (3.31–4.44)	–	–
Diuron (pure active ingredient) (mg L^{-1})	94.6 (84.9–104.4)	81.0 (70.8–91.2)	73.6 (66.4–80.8)	64.6 (57.7–71.6)	0.61 (0.35–0.87)	7.10 (5.90–8.29)
Diuron (Diuron Nortox® 500 SC) (mg L^{-1})	154.6 (138.0–171.2)	112.7 (101.5–123.8)	78.3 (71.6–84.9)	62.4 (57.1–67.7)	0.33 (0.09–0.58)	6.78 (4.88–8.67)
Carbofuran (pure active ingredient) (mg L^{-1})	239.3 (233.7–244.9)	200.6 (191.4–209.7)	174.0 (163.3–184.8)	142.0 (136.9–147.0)	0.30 (0.02–0.58)	22.95 (15.60–30.30)
Carbofuran (Furadan® 350 SC) (mg L^{-1})	170.0 (167.7–172.4)	119.7 (116.4–123.0)	98.5 (95.0–102.0)	70.4 (65.9–75.0)	0.21 (0.06–0.35)	4.98 (3.70–6.27)

caused a significant growth inhibition in populations exposed to concentrations of 1.75–28 mg L^{-1}, as evidenced by Dunnett's test (pure active ingredient diuron: $F_{6,21} = 352.9$, $p = 0.00002$; commercial product diuron: $F_{6,21} = 810.8$, $p = 0.00002$). Regarding growth inhibition, the highest concentration of the diuron (pure active ingredient and commercial product) without any observable effect (NOEC) was 0.88 mg L^{-1}, while the lowest concentration causing an observable effect (LOEC) was 1.75 mg L^{-1}. For carbofuran, a significant growth inhibition was observed in populations of *P. caudatum* exposed to concentrations in the range of 1.88–60 mg L^{-1} of carbofuran in its pure form (Fig. 2C) and 0.94–15 mg L^{-1} of carbofuran dosed as Furadan® 350 SC (Fig. 2D), as evidenced by the results of the Dunnett's test (pure active ingredient carbofuran: $F_{6,21} = 236.8$, $p = 0.00002$; commercial

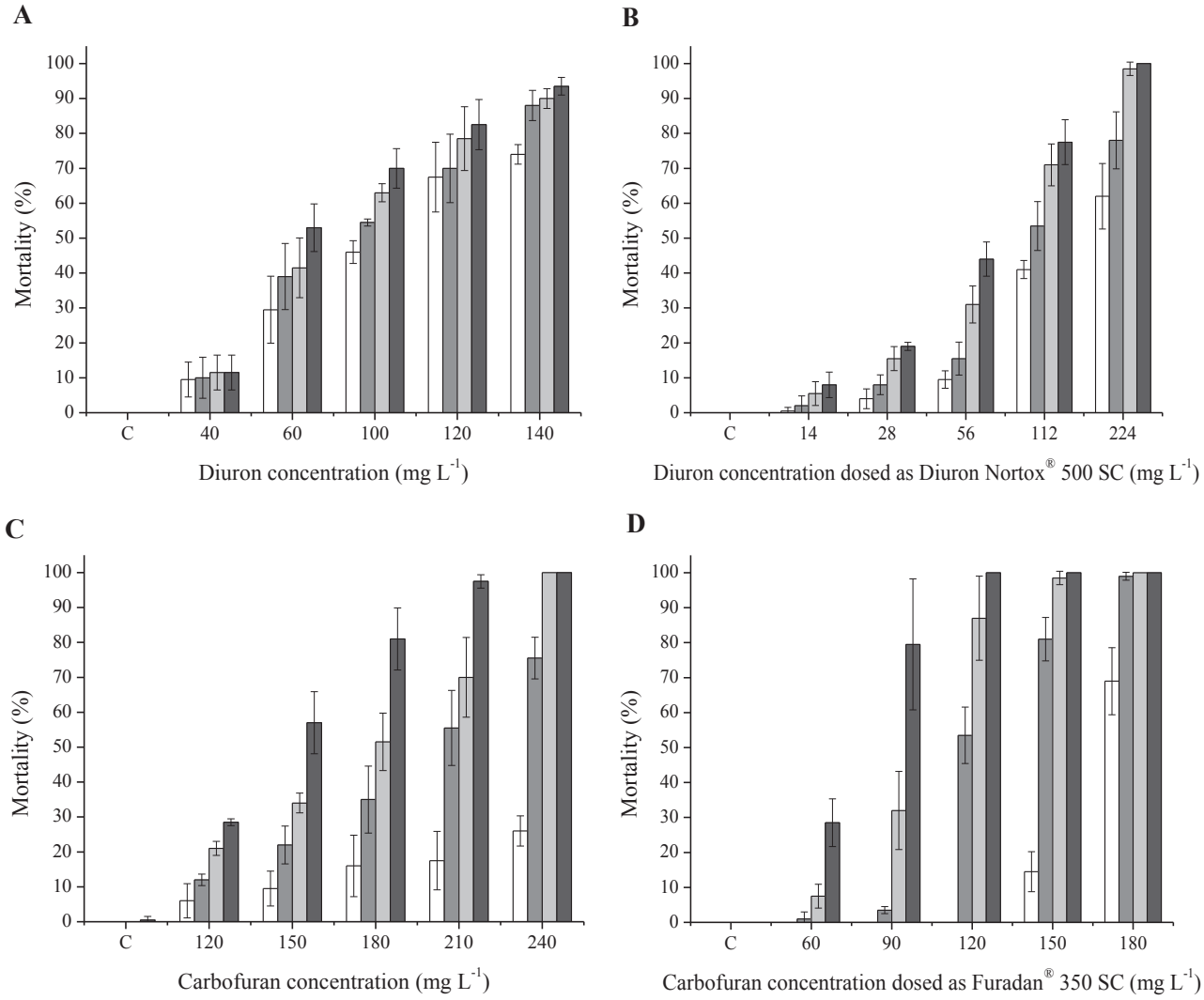

Fig. 1. Mortality (%) of *P. caudatum* at different exposure periods (2, 3, 4 and 6 h) to different concentrations of diuron in its pure form (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran in its pure form (C) and carbofuran dosed as Furadan® 350 SC (D).

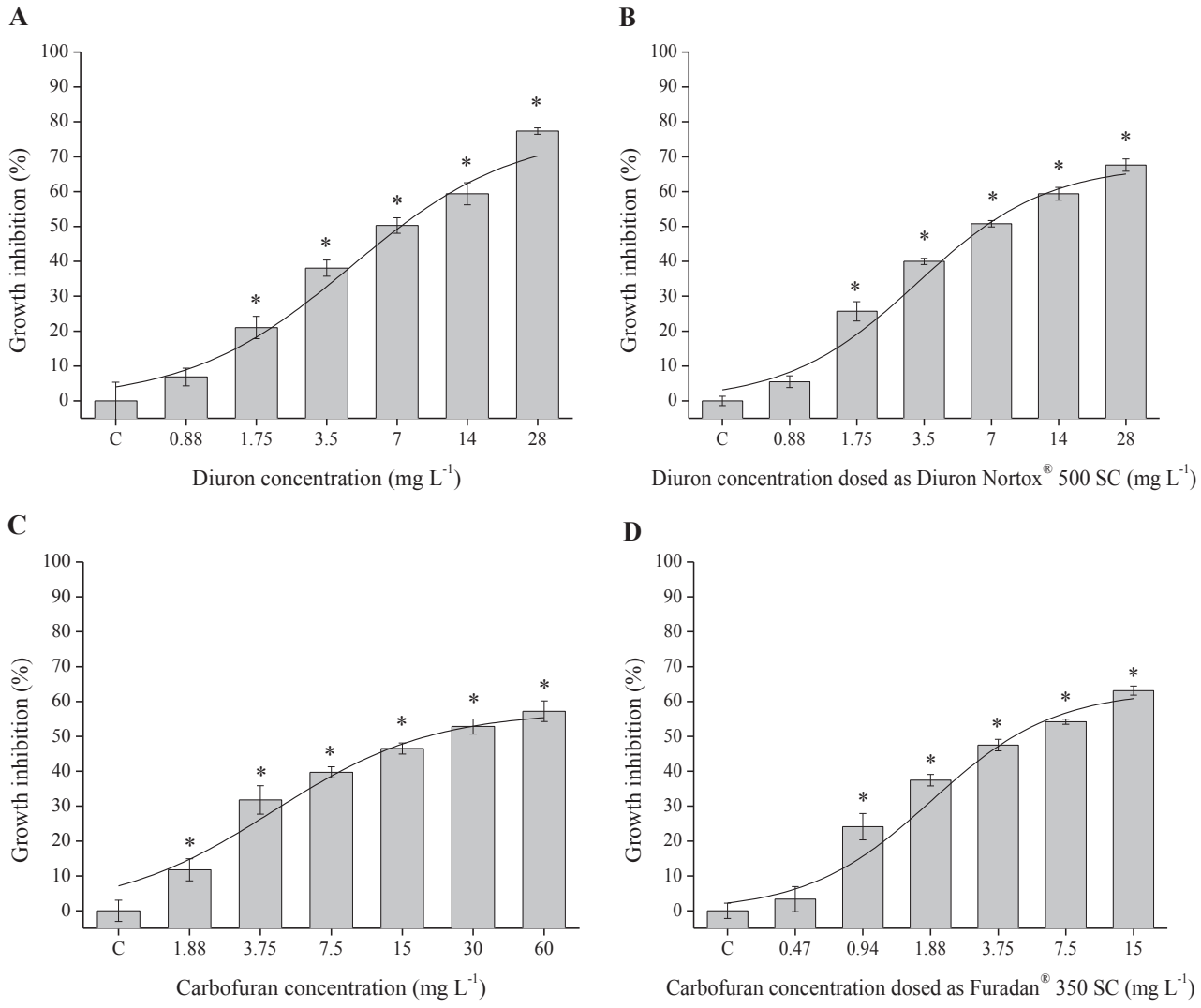

Fig. 2. Population growth inhibition (%) of *P. caudatum* exposed to different concentrations of diuron in its pure form (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran in its pure form (C) and carbofuran dosed as Furadan® 350 SC (D). The line represents the logistic curve-fitting. The symbol * indicates significant difference from control at $p < 0.05$.

product carbofuran: $F_{6,21} = 426.5$, $p = 0.00002$). The NOEC of carbofuran was <1.88 mg L^{-1} and 0.47 mg L^{-1} for pure active ingredient and commercial product, respectively. The LOEC of carbofuran was 1.88 mg L^{-1} and 0.94 mg L^{-1} for pure active ingredient and commercial product, respectively.

The results of the effects of the tested pesticides on the number of generations of *P. caudatum* are presented in Fig. 3. All compounds tested showed concentrations that significantly reduced the replication numbers, compared to the control: pure active ingredient diuron: $F_{6,21} = 494.6$, $p < 0.05$; diuron dosed as Diuron Nortox® 500 SC: $F_{6,21} = 659.9$, $p < 0.05$; pure active ingredient carbofuran: $F_{6,21} = 192.6$, $p < 0.05$; carbofuran dosed as Furadan® 350 SC: $F_{6,21} = 521.0$, $p < 0.05$. For diuron, both pure active ingredient (Fig. 3A) and commercial product (Fig. 3B), the lowest concentration that caused a significant reduction of number of generations was 1.75 mg L^{-1}. For carbofuran, the lowest concentration tested that caused a significant reduction of the number of generations was 1.88 mg L^{-1} of carbofuran in its pure form (Fig. 3C) and 0.94 mg L^{-1} of Furadan® 350 SC (Fig. 3D). Based on the logistic curve-fitting, the EC_{10} values for generation number were: 1.54 mg L^{-1} (0.93–2.15) of pure diuron; 0.62 mg L^{-1} (0.30–0.93) of commercial diuron; 0.77 mg L^{-1} (0.19–1.36) of pure carbofuran and 0.40 mg L^{-1} (0.20–0.60) of commercial carbofuran.

Fig. 4 shows the effect of pesticides on *P. caudatum* biomass. All compounds tested presented concentrations that significantly reduced the biomass, compared to control (pure active ingredient diuron: $F_{6,203} = 512.6$, $p < 0.05$; diuron dosed as Diuron Nortox® 500 SC: $F_{6,203} = 533.7$, $p < 0.05$; pure active ingredient carbofuran: $F_{6,203} = 438.7$, $p < 0.05$; carbofuran dosed as Furadan® 350 SC: $F_{6,203} = 661.6$, $p < 0.05$). For diuron, both pure active ingredient (Fig. 4A) and commercial product (Fig. 4B), the lowest concentration that caused a significant decrease of the biomass was 1.75 mg L^{-1}. For carbofuran, the lowest concentration that caused a significant decrease of biomass was 1.88 mg L^{-1} of carbofuran in its pure form (Fig. 4C) and 0.47 mg L^{-1} of carbofuran dosed as Furadan® 350 SC (Fig. 4D). From the logistic curve-fitting, the EC_{10} values for protozoan biomass reduction were: 1.08 mg L^{-1} (0.85–1.31) of pure diuron; 0.52 mg L^{-1} (0.38–0.67) of commercial diuron; 1.43 mg L^{-1} (1.02–1.84) of pure carbofuran and 0.14 mg L^{-1} (0.09–0.19) of commercial carbofuran.

3.4. Preliminary risk assessment

According to the FOCUS model (step 2), diuron presented a

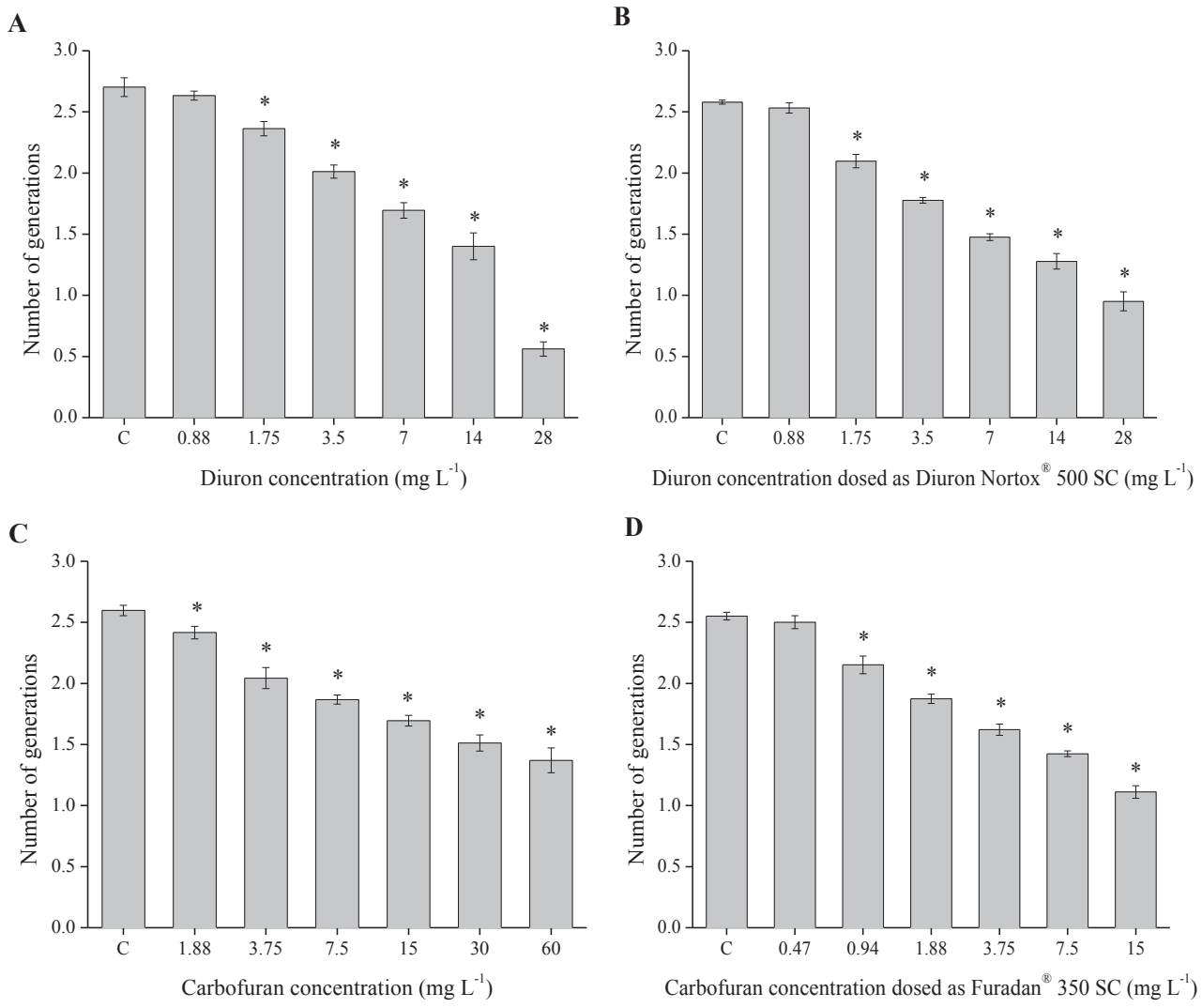

Fig. 3. Effects on number of generations of *P. caudatum* exposed to different concentrations of diuron in its pure form (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran in its pure form (C) and carbofuran dosed as Furadan® 350 SC (D). The symbol * indicates significant difference from control at $p < 0.05$.

maximum PEC in the surface water of 0.293 mg L^{-1} and a time-weighted average PEC after 24 h of maximum peak (24 h-PECtwa) of 0.281 mg L^{-1}. For carbofuran, the maximum PEC value was 0.193 mg L^{-1} and 24 h-PECtwa value was 0.182 mg L^{-1}. By using the acute toxicity data for PNECs calculation ($PNEC_{diuron}$ = 0.065 mg L^{-1}; $PNEC_{carbofuran}$ = 0.142 mg L^{-1}), RQ values were 4.5 and 1.4 for diuron and carbofuran, respectively. When chronic toxicity data were used as basis for producing the corresponding PNECs ($PNEC_{diuron}$ = 0.006 mg L^{-1}; $PNEC_{carbofuran}$ = 0.003 mg L^{-1}), RQ values were 46.1 and 60.7 for diuron and carbofuran, respectively (Supplemental Table S1). According to the results, all RQ values were >1, indicating that pesticides diuron and carbofuran represent potential ecological risks for Brazilian water bodies.

4. Discussion

Our results showed that *P. caudatum* was considerably sensitive to sodium chloride, a commonly used reference substance (Environmental Canada, 1990; USEPA, 2002), being more sensitive than the algae *Chlamydomonas reinhardtii* (Moser and Bell, 2011), the cladoceran *Daphnia pulex* (Bezirci et al., 2012), the amphipod *Gammarus roeseli* (Sornom et al., 2010) and the larvae of the insect *Chironomus dilutes* (Elphick et al., 2011). Sensitivity differences are expected based on species intrinsic characteristics and differences in the test conditions, as reported by other authors (e.g. Freitas and Rocha, 2012; Moreira et al., 2014).

Freshwater protozoan *P. caudatum* possess a contractile vacuoles complex, responsible for controlling the cytosolic osmolarity and the water permeability of the plasma membrane, allowing osmoregulation under hypertonic conditions (Allen and Naitoh, 2002; Stock et al., 2002). According to Smurov and Fokin (1999), freshwater ciliates are capable of regulating salt internal ionic composition, probably, up to 5.0 g L^{-1}. In the present study, the concentration of 5.05 g NaCl L^{-1} caused 50% mortality of *P. caudatum* individuals after 2 h exposure and this value was reduced to 3.88 g L^{-1} after 6 h exposure. Based on our results, this reference substance was considered adequate to assess the state of health of protozoans before conducting the toxicity tests.

Although conventionally the exposure time adopted in ecotoxicological studies is previously determined, our results evidenced that variable exposure times (2, 3, 4 and 6 h) greatly alters the toxic response of *P. caudatum* to the pesticides tested, and that mortality increases steadily with the increase in the exposure time (Fig. 1).

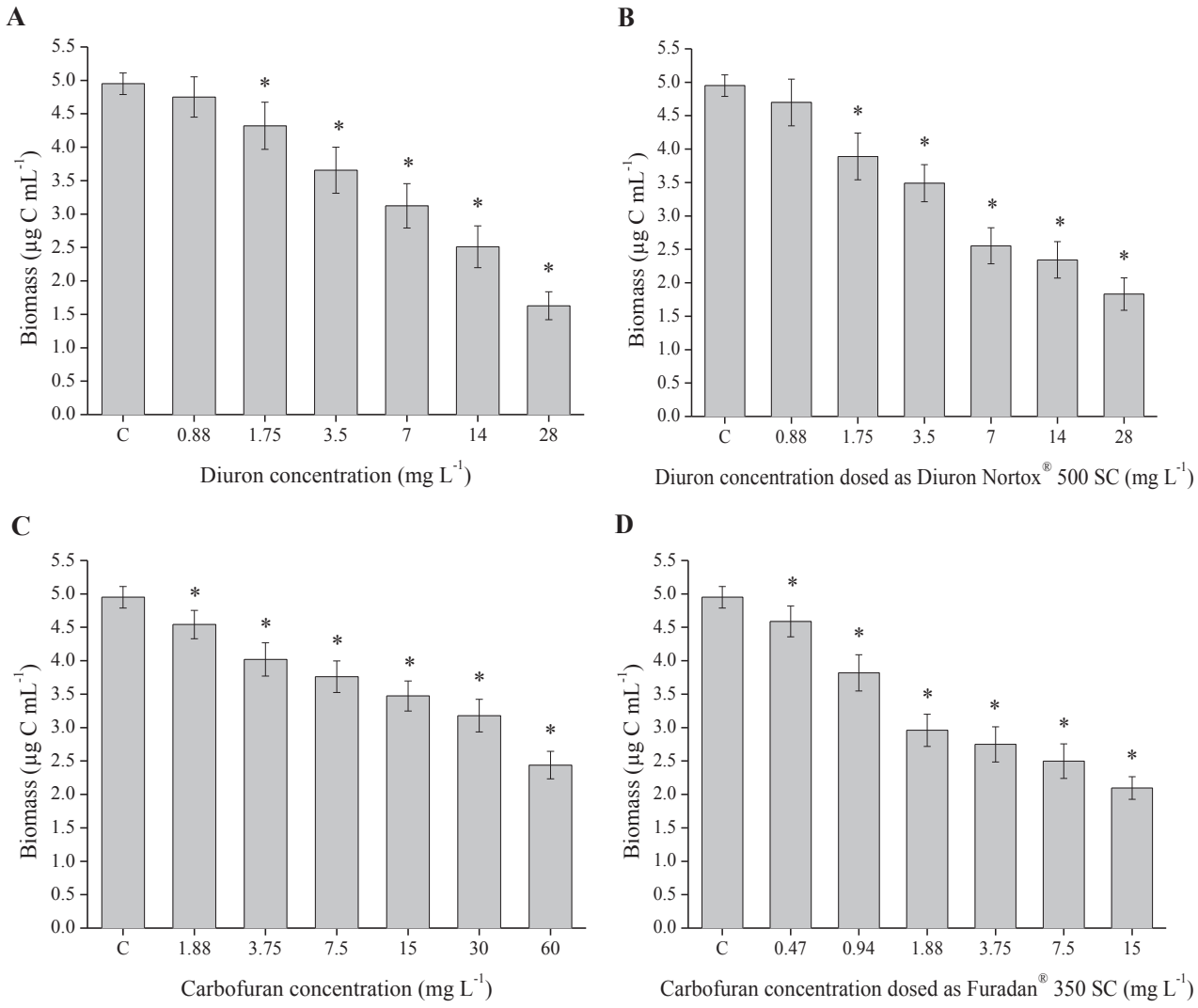

Fig. 4. Effects on biomass (µg C mL^{-1}) of *P. caudatum* exposed to different concentrations of diuron in its pure form (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran in its pure form (C) and carbofuran dosed as Furadan® 350 SC (D). The symbol * indicates significant difference from control at $p < 0.05$.

The toxicity of diuron and carbofuran (as pure active ingredient) was, respectively, 1.5 and 1.7 times higher after 6 h of exposure than after 2 h. For diuron dosed as Diuron Nortox® 500 SC and carbofuran dosed as Furadan® 350 SC toxicity was 2.5 and 2.4 times, respectively, higher after 6 h of exposure than after 2 h (see Table 1). Some authors have also reported toxicity variation dependent on exposure time for protozoans (Zhou et al., 2011) and other organisms, such as cladocerans (Gama-Flores et al., 2008) and fishes (Mohanty et al., 2013).

Comparing the toxicity of the active ingredient diuron in its pure form with that of its commercial formulation, the 6 h LC_{50} and the 24 h IC_{50} mean values showed that, considering the 95% confidence interval, their toxicity to *P. caudatum* were similar, indicating that the toxic potency of the active ingredient in the commercial product Diuron Nortox® 500 SC was not affected by the substances added. However, the sensitivity of this species to carbofuran in its commercial formulation, the Furadan® 350 SC, was higher than to its pure active ingredient (mortality and growth inhibition were 2.0 and 4.6 times higher, respectively), suggesting that the increased toxicity of the commercial product resulted from synergistic effects of the other ingredients added to the carbofuran.

Commercial pesticide formulations contain the active ingredient mixed with other chemicals (generally called “inert” ingredients) that act as solvents, emulsifiers, surfactants and/or preservatives (Cox and Surgan, 2006). Several authors (e.g. Krogh et al., 2003; Oakes and Pollak, 2000; Solomon and Thompson, 2003) have shown that the so-called inert ingredients can contribute to the toxicity of the formulation, either by exerting toxic activity on their own, or by interacting with the active ingredient. In many studies the toxicity of the commercial formulations is higher than that of the active ingredient (e.g. Beggel et al., 2010; Cedergreen and Streibig, 2005; Pereira et al., 2009) and as found in the present study for the commercial carbofuran. However, this is not always the case, as verified for the toxicity of the commercial form of the diuron to *P. caudatum* and also by other authors. Pessoa et al. (2011) when comparing the toxicity of carbofuran and Furadan® 350 SC to *Oreochromis niloticus* fish larvae found similar 96-h LC_{50} values for these compounds, thus indicating that the toxic potency of the active ingredient was not affected by the inert substances added in the commercial formulation. Kroon et al. (2015) verified that estrogenic biomarkers in *Lates calcarifer* fish juveniles increased following exposure to diuron commercial formulation (Diurex® WG) but not to the pure chemical, suggesting an estrogenic response to the additives.

Therefore, tests with active ingredient in its pure form and also with its commercial formulation should be tested to a variety of species to provide more realistic information on the pesticide toxicity that may cause different responses, both due to the associated inert ingredients as toxicity answers be usually species-specific.

The comparison of *P. caudatum* 6 h LC_{50} values with those from a wide variety of species showed that this protozoan is less sensitive to diuron (pure active ingredient) than all the organisms presented in Table 2. However, when the 24 h IC_{50} values were compared, *P. caudatum* was more sensitive than *Vibrio fischeri* bacterium, *T. pyriformis* protozoan, *Aiptasia* sp. anemone, *Hydroides elegans* polychaete, *Daphnia magna* cladoceran, *Artemia salina* anostraca, *Balanus amphitrite* barnacle and the *Psetta maxima* fish (embryo-larvae) (see Table 2). Due to the specific action mode of herbicide diuron, the photosynthetic organisms (cyanobacteria, algae and macrophyte) were the most sensitive. However, diuron also cause toxic effects in heterotrophic non-target organisms (invertebrates, amphibians and fishes) by different modes of action, for example AChE activity inhibition (Ahmed et al., 2012; Bretaud et al., 2000), teratogenic effects (Lazhar et al., 2012), endocrine disrupting (Noguerol et al., 2006; Orton et al., 2009) and immunotoxic effects (Luna-Acosta et al., 2012).

Although the mode of action of diuron is well established for a variety of species, its mode of action for protozoans is still unclear. From the results regarding the acute toxicity of diuron on *P. caudatum*, it was verified that this compound lysed the plasmatic membrane of this protozoan at the highest concentrations tested. Some studies demonstrated that diuron exert toxic effects on heterotrophic microorganisms, such as yeast *Saccharomyces cerevisiae,* blocking the mitochondrial respiratory chain (Dragone et al., 2015; Estève et al., 2009) and generating reactive oxygen species (Tenda et al., 2012). Reactive oxygen species (ROS) are naturally generated as a consequence of cell metabolism, but when it comes in contact with exogenous agents such as herbicides, the ROS level may increase with subsequent induction of oxidative stress. ROS react with lipids and in this case it leads to aldehyde formation causing membrane disintegration and consequent cell death (Catalá, 2006; Tenda et al., 2012). Thus, in this study, the diuron toxicity on *P. caudatum* was probably due to inhibition of respiration and ROS level increase with consequent cell lysis.

Diuron caused significant reduction on *P. caudatum* population growth from the concentration of 1.75 mg L^{-1} Bricheux et al. (2013), using the microcalorimetry technique, showed that the metabolic activity and growth of protozoan *T. pyriformis* is progressively inhibited with the increase in the diuron concentrations and revealed that the general state of the living cell was severely altered at a concentration of 56.0 mg L^{-1}. The *T. pyriformis* IC_{50} value estimated by these researchers was 13.8 mg L^{-1} by microcalorimetry and 18.6 mg L^{-1} by flow cytometry.

In this study, the population growth inhibition test (IC_{50}) responded potentially better than the acute test which was based on mortality (LC_{50}), because the population growth test integrates all the effects at various levels of cellular metabolism and thus, makes it possible to assess a global effect on a series of processes implied in cellular division. Thus, the determination of the toxicity

Table 2
Toxicity values (LC_{50}, IC_{50} or EC_{50}) obtained from the literature to a broad spectrum of species of different taxonomic groups exposed to diuron and carbofuran pesticides.

Group	Species	Endpoint (mg L^{-1})	Pesticide	Reference
Bacteria	*Vibrio fischeri*	9.20 (30 min EC_{50})	Diuron	Gatidou et al. (2015)
		58.07 (30 min EC_{50})	Diuron	Bonnet et al. (2007)
		28.90 (30 min EC_{50})	Carbofuran	Fernández-Alba et al. (2002)
Cyanobacteria	*Anabaena flos-aquae*	7.93 (96 h IC_{50})	Carbofuran	Ma et al. (2006)
Cyanobacteria	*Microcystis aeruginosa*	4.65 (96 h IC_{50})	Carbofuran	Ma et al. (2006)
Algae	*Achnanthidium minutissimum*	0.06 (96 h IC_{50})	Diuron	Larras et al. (2013)
Algae	*Craticula accomoda*	1.43 (96 h IC_{50})	Diuron	Larras et al. (2013)
Algae	*Skeletonema costatum*	0.006 (96 h IC_{50})	Diuron	Bao et al. (2011)
Algae	*Chlorella vulgaris*	7.86 (96 h IC_{50})	Carbofuran	Ma et al. (2006)
Algae	*Dunaliella tertiolecta*	0.009 (96 h EC_{50})	Diuron	DeLorenzo et al. (2013)
Algae	*Raphidocelis subcapitata*	6.22 (96 h IC_{50})	Carbofuran	Ma et al. (2006)
		0.022 (72 h IC_{50})	Diuron	Zhang et al. (2012)
Macrophyte	*Lemna minor*	0.028 (168 h IC_{50})	Diuron	Gatidou et al. (2015)
Protozoan	*Paramecium caudatum*	210.0 (3 h LC_{50})	Carbofuran	Hussain et al. (2008)
Protozoan	*Tetrahymena pyriformis*	13.80 (15 h IC_{50})	Diuron	Bricheux et al. (2013)
		7.84 (9 h IC_{50})	Diuron	Bonnet et al. (2007)
		>100 (1 h IC_{50})	Diuron	Bogaerts et al. (2001)
Rotifer	*Brachionus calyciflorus*	14.10 (24 h LC_{50})	Carbofuran	Iesce et al. (2006)
Rotifer	*Philodina roseola*	13.36 (48 h EC_{50})	Carbofuran	Moreira et al. (2015)
Cnidarian	*Aiptasia* sp.	19.00 (96 h LC_{50})	Diuron	Bao et al. (2011)
Cnidarian	*Acropora tumida*	4.80 (24 h LC_{50})	Diuron	Bao et al. (2011)
Polychaete	*Hydroides elegans*	16.00 (48 h LC_{50})	Diuron	Bao et al. (2011)
Cladoceran	*Ceriodaphnia dubia*	0.003 (96 h LC_{50})	Carbofuran	Bailey et al. (1996)
Cladoceran	*Daphnia magna*	8.60 (48 h EC_{50})	Diuron	Hernando et al. (2005)
		0.018 (48 h EC_{50})	Carbofuran	Hernando et al. (2005)
		0.059 (48 h EC_{50})	Carbofuran	Herbrandson et al. (2003)
Copepod	*Nitocra spinipes*	4.00 (96 h EC_{50})	Diuron	Karlsson et al. (2006)
Anostracod	*Artemia salina*	12.01 (24 h LC_{50})	Diuron	Koutsaftis and Aoyama (2007)
Amphipod	*Gammarus pulex*	0.009 (24 h LC_{50})	Carbofuran	Ashauer et al. (2010)
Barnacle	*Balanus amphitrite*	21.00 (24 h LC_{50})	Diuron	Bao et al. (2011)
Water mite	*Arrenurus* sp.	7.00 (96 h EC_{50})	Diuron	James (2008)
Insect	*Anopheles stephensi*	0.26 (24 h LC_{50})	Carbofuran	Kumar et al. (2004)
Insect	*Chironomus riparius*	0.027 (24 h LC_{50})	Carbofuran	Ibrahim et al. (1998)
Fish	*Clarias batrachus*	23.00 (96 h LC_{50})	Carbofuran	Begum (2008)
Fish	*Danio rerio*	1.34 (24 h LC_{50})	Carbofuran	Lee et al. (2014)
Fish	*Oreochromis niloticus*	0.21 (96 h LC_{50})	Carbofuran	Pessoa et al. (2011)
Fish	*Pimephales promelas*	1.99 (96 h LC_{50})	Carbofuran	Tarkowski (2004)
Fish	*Psetta maxima*	7.83 (96 h LC_{50})	Diuron	Mhadhbi and Beiras (2012)

of a compound on ciliated protozoan using the growth inhibition endpoint compared with the mortality seems to be of great importance, as also evidenced by Gomiero et al. (2013).

A comparison of the sensitivity of *P. caudatum* to carbofuran (pure active ingredient) to a variety of species using the 6 h LC_{50} values showed that this protozoan is less sensitive than all the organisms presented in Table 2. When it was compared the 24 h IC_{50} values, *P. caudatum* was more sensitive than the bacteria *V. fischeri* and the fish *Clarias batrachus*. Hussain et al. (2008) reported that the pesticide carbofuran caused inhibitory effect on phagocytosis (19.71% reduction in food vacuole formation at 135 mg L^{-1}) and on the pulsatory vacuole activity (87–135 mg L^{-1}) to *P. caudatum*. The 3 h LC_{50} value found by these authors for carbofuran was 210 mg L^{-1}, while in our study the value found was 200.6 mg L^{-1} (191.4–209.7) (Table 1).

The low sensitivity of *P. caudatum* to the pesticides tested evidenced by mortality data (LC_{50}) could be explained by the bioaccumulation of compounds in the cell as a resistance mechanism. Many studies recognize that microorganisms have a high affinity for metals and toxic xenobiotics and can accumulate both heavy metals and toxic xenobiotics by a variety of mechanisms (e.g. Lal et al., 1987; Mortimer et al., 2010; Pas et al., 2004; Rehman et al., 2008; Yilmaz, 2003). Martín-González et al. (2005) and Mortimer et al. (2010) showed that ciliated protozoa can sequester metals by ingesting it into the food vacuoles or by accumulating excessive metal ions in cytoplasmic dense granules. In our study, during the acute toxicity tests, we observed that *P. caudatum* probably accumulated the pesticides diuron and carbofuran into the food vacuoles (Supplemental Fig. S4).

Under stressful conditions, such as exposure to pollutants, energy reserves are used in detoxification processes, and consequently, protozoa inhabiting polluted environments can display reduced replication and growth rates (Gomiero et al., 2013). Our results showed that both the diuron as carbofuran significantly reduced the growth (Fig. 2) and the number of generations (cell divisions) of *P. caudatum* (Fig. 3). The occurrence of pollutant's concentrations capable of inducing alterations on reproduction mechanisms, such as the replication rate, may severely modify natural community structure in terms of the species occurrence and population relative abundances (Gomiero and Viarengo, 2014).

An alteration in the protozoan-component of microbial communities caused by the toxic effects of contaminants can alter the trophic chain and significantly affect the environmental balance (Gomiero et al., 2013; Trielli et al., 2007). Our results revealed that the pesticides diuron and carbofuran significantly reduced the protozoan biomass at the lesser concentrations tested (see Fig. 4) due to effects on growth and reproduction of *P. caudatum*. Li et al. (2015) reported that the biomass of *Tetrahymena thermophila* decreased in high concentrations (100, 1000 or 10,000 μM of tris (1,3-dichloro-2-propyl) phosphate) after 8-h exposure, but when exposure time was lengthened to 5 days lesser-concentrations exposure (0.1 or 1 μM) caused similar effects. These authors suggested that contaminants can cause toxic effects at lower concentrations in ciliated protozoan by multi-generation exposure, possibly threatening wildlife upon exposure at environmental-relevant concentrations.

In this study, relevant ecological information were obtained by combining the results from the mortality, population growth inhibition, decrease of the number of generations and biomass, since these effects on protozoans are related to important structural and functional mechanisms that can bring adverse effects to aquatic ecosystems. Therefore, the incorporation of multiple ecologically relevant endpoints in the ecotoxicological tests used in routine environmental management programs seems to be a pragmatic way to link environmental fates with their causes (Galloway et al., 2004).

The short life-cycle of *P. caudatum* allowed the analysis of the chronic toxic effects of the pesticides on a conspicuous number of cells and for several generations (±2.6 generations), with high degree of reproducibility and quick responses (24 h). Many studies have shown that the protozoan *P. caudatum* is a good test organism to evaluate the toxic effects caused by metal pollution (Gong et al., 2014; Kvitek et al., 2009; Madoni, 2011; Wu et al., 2015). However, studies with this organism to evaluate the effect of pesticides are still scarce (e.g. Miyoshi et al., 2003). This study revealed that *P. caudatum* is more sensitive to the pesticide diuron than other species commonly used in standard ecotoxicological studies, such as *V. fischeri*, *Daphnia magna* and *A. salina*. Therefore, this ciliated protozoan fulfilled several requirements to be used as test organism in ecotoxicological studies and confirmed that it is a suitable test organism to evaluate pesticides toxicity on aquatic environments.

Preliminary risk assessment of the pesticides diuron and carbofuran showed RQs >1, indicating these compounds present potential ecological risks on water bodies in Brazil. Based on data obtained, chronic toxic effects on *P. caudatum* are likely to occur in many aquatic environments. However, this preliminary risk assessment must be viewed with caution, because it was carried out using ecotoxicological data of only one species (*P. caudatum*), thus corresponding to a single trophic level. To refine the risk assessment, ecotoxicological data from at least three species (normally algae, *Daphnia* and fish) representing three trophic levels, should be included (European Commission, 2011), what constitutes a relevant subject to near-future research.

For future studies, we also suggest that the sublethal effects of carbofuran and diuron on protozoans and its ecological implications on aquatic ecosystems should be evaluated in experiments using micro and mesocosms (more complex and realistic set-ups), which can provide insights for a deeper understanding of the effects of these pesticides and of its interactions with environmental factors in the aquatic systems.

5. Conclusion

Our study revealed that both diuron and carbofuran pesticides cause deleterious effects on ciliated protozoans as evidenced by *P. caudatum* responses in this study. In the conditions and concentrations tested and for this particular test organism, a unicellular ciliate protozoan, the toxicity of the pure active ingredient of diuron and of its commercial form was similar, thus evidencing that the "inert" ingredients did not have synergistic effects. On the other hand, the acute toxicity of carbofuran in its commercial formulation was two times more toxic than its active ingredient alone, suggesting the occurrence of synergistic interactions with the inert ingredients and corroborating the general finding that toxicity is compound-specific. The sublethal responses adopted for evaluating chronic toxicity, such as population growth inhibition, number of generations and biomass were sensitive and adequate to show the extent in which these pesticides can affect non-target aquatic organisms. Despite their structural simplicity, ciliate protozoans as *P. caudatum*, are sensitive and display a set of identifiable responses to diuron and carbofuran toxicity, both at individual and population levels, thus being adequate test organism. Although the concentrations that caused deleterious chronic effects were relatively high at short exposure, with lengthened exposure times involving multiple generations, toxic effects can occur at much lower concentrations approaching more realistic environmental conditions. The preliminary risk assessment performed suggests that the pesticides here tested deserve greater attention regarding their use, by presenting real threats to many non-target organisms in Brazilian water bodies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.054.

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