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# Effects of diuron and carbofuran and their mixtures on the microalgae *Raphidocelis subcapitata*

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ABSTRACT

In aquatic environments, organisms are often exposed to mixtures of several pesticides. In this study, the effects of carbofuran and diuron and their mixtures on the microalgae *Raphidocelis subcapitata* were investigated. For this purpose, toxicity tests were performed with the single compounds (active ingredients and commercial formulations) and their combinations (only active ingredients). According to the results, the toxicity of active ingredients and their commercial formulations to *R. subcapitata* was similar. In the single exposures, both carbofuran and diuron inhibited significantly the *R. subcapitata* growth and caused physiological (chlorophyll *a* content) and morphological (complexity and cell size) changes in cells, as captured by flow cytometry single-cell properties. Regarding the mixture toxicity tests, data fitted to both reference models, concentration addition (CA) and independent action (IA), and evidenced significant deviations. After the CA fitting, dose-ratio dependent deviation had the best fit to the data, demonstrating synergism caused mainly by diuron and antagonism caused mainly by carbofuran. After fitting the IA model, a synergistic deviation represented the best fit for the diuron and carbofuran mixtures. In general, the two reference models indicated the occurrence of synergism in the mixtures of these compounds, especially when diuron was the dominant chemical in the combinations. The increased toxicity caused by the mixture of these pesticides could pose a greater environmental risk for phytoplankton. Thus, exposure to diuron and carbofuran mixtures must also be considered in risk assessments, since the combination of these compounds may result in more severe effects on algae population growth than single exposures.

## 1. Introduction

Aquatic environments are often contaminated with pesticides from different sources, mainly from agriculture runoff, constituting a potential hazard to non-target organisms. These organisms are rarely exposed to a single contaminant, but usually to mixtures of several pesticides with varying constituents in different concentrations and ratios (Faust et al., 2003; Schuler and Rand, 2008). Many pesticides are persistent and their continued and increasing use represents a major threat to aquatic environments through acute and chronic exposure (Faust et al., 2001; McClellan et al., 2008) and their mixtures may lead to additive effects or produce more severe (synergistic) or less severe (antagonistic) effects (Liu et al., 2013; Magnusson et al., 2010).

Risk assessments for regulation of chemicals and most ecotoxicological studies in aquatic environments have focused mainly on the single compounds toxicity under controlled conditions (Barata et al., 2006). However, considering that organisms in the ecosystem are constantly exposed to complex mixtures of toxic substances (Cedergreen et al., 2008; Ferreira et al., 2008; Pavlaki et al., 2011), some tools were developed (Cassee et al., 1998; Jonker et al., 2005) to predict and evaluate in a more realistic way the contaminants behavior when they occur together in the environment.

Theoretical models used the toxicity of mixtures based on two non-interaction concepts, concentration addition (CA) (Loewe and Muischnek, 1926) and independent action (IA) (Bliss, 1939). The CA model assumes that individual chemicals have the same mode of action and act upon the same biological target, contributing to a common response in proportion to their respective toxicities (Ferreira et al., 2008; Freitas et al., 2014; Loureiro et al., 2010). On the other hand, the IA model assumes that individual chemicals have different modes of action and their effects are therefore statistically independent of each other (Ferreira et al., 2008; Freitas et al., 2014; Loureiro et al., 2010).

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When modes of action of the chemicals are unknown, both CA and IA models are applied and the one that best fits the data is chosen over the other (Pavlaki et al., 2011).

In real conditions, compounds can interact with each other, modifying the amplitude and sometimes the nature of the toxic effect. Interactions may occur in the toxicokinetic phase (processes of uptake, distribution, metabolism and excretion) or in the toxicodynamic phase (effects of chemicals on the receptor, cellular target or organ) (Cassee et al., 1998). Thus, the interaction between pesticides might result in deviations from the CA and IA models. The deviations expected are those that produce synergism or antagonism, or dose-ratio dependent (deviations vary according to mixture composition) or dose-level dependent (different deviations at high and low concentrations) (Ferreira et al., 2008; Jonker et al., 2005).

Diuron and carbofuran are commonly used pesticides in diverse crops, such as cotton, coffee, sugar cane, corn, wheat, and have often been found in water bodies worldwide (e.g. Faggiano et al., 2010; Kaonga et al., 2015; Masiá et al., 2015; Papadakis et al., 2015), including Brazil (e.g. Caldas et al., 2011; Carbo et al., 2008; Dantas et al., 2011; Loro et al., 2015). Diuron (phenylurea) is a herbicide that inhibits photosynthesis by blocking the electron transport chain at the photosystem II in microorganisms and photosynthetic plants (Giacomazzi and Cochet, 2004), whereas carbofuran (carbamate) is an insecticide, acaricide and nematicide that acts against a wide variety of organisms by binding to enzyme acetylcholinesterase, inhibiting its action on the acetylcholine (Pessoa et al., 2011).

Contamination of aquatic environments with diuron and carbofuran may induce adverse effects on organisms, including microalgae. Algae play a key role in aquatic ecosystems because, as primary producers, are an important part at the base of the food web and any effect on them may affect the higher trophic levels and consequently impact the ecosystem functioning (DeLorenzo et al., 2002; Rioboo et al., 2007). For this reason, algal species are often used in risk assessments of chemicals (Pérez et al., 2011; Ribeiro et al., 2014). In addition to their important ecological role, microalgae are easy to cultivate, have a short generation time and are sensitive to several compounds (e.g. herbicides), which makes them suitable biological tools in ecotoxicological testing for pollutants (e.g. Stachowski-Haberkorn et al., 2013; Suman et al., 2015).

The aim of this study was to evaluate the effects of diuron and carbofuran and their mixtures on microalgae *Raphidocelis subcapitata*. For this purpose, toxicity tests were performed with single compounds (active ingredients and commercial formulations) and their binary combinations (only for active ingredients) and analyzed population growth rate and single-cell properties, such as chlorophyll *a* content, cell size and complexity by flow cytometry.

## 2. Materials and methods

### 2.1. Test organism and culture conditions

A *Raphidocelis subcapitata* strain was obtained from stock cultures of the Ecotoxicology Laboratory at the Federal University of São Carlos, SP, Brazil. Cultures of the microalga were maintained in LC Oligo medium (AFNOR – Association Française de Normalisation, 1980) under continuous illumination (4306 lx), controlled temperature (25 ± 1 °C) and manual agitation three times a day. The algal cells used in the assay were three days old (exponential growth phase, data not shown).

### 2.2. Chemicals and test solutions

Diuron (CAS no 330-54-1) and carbofuran (CAS no 1563-66-2), both of high purity (≥98%, analytical standard), were purchased from Sigma-Aldrich. The purity level of the commercial formulation Diuron Nortox® 500 SC (purchased from Nortox S/A, Brazil) is 50% m/v of active ingredient (69.4% m/v of inert ingredients) and of the Furadan® 350 SC (purchased from FMC, Brazil) is 35% m/v of active ingredient (65% m/v of inert ingredients). The stock solutions of diuron dosed as Diuron Nortox® 500 SC (100 mg a.i. $L^{-1}$), carbofuran (100 mg a.i. $L^{-1}$) and carbofuran dosed as Furadan® 350 SC (100 mg a.i. $L^{-1}$) were prepared by dilution of a specific amount of each compound in distilled water immediately before the tests, with exception of diuron, which was dissolved in acetonitrile (≥99.9%, HPLC grade) due to its low solubility in water (42 mg $L^{-1}$ at 20 °C). In turn, the nominal concentrations of each substance tested were obtained by dilution of the stock solution in culture medium (LC Oligo).

To confirm the nominal concentrations used in the tests, stock solutions and test concentrations were quantified using an Agilent Technologies series 1200 high-performance liquid chromatograph (HPLC) (Waldbronn, Germany), equipped with a diode array detector (DAD). The chromatographic analytical conditions were the same described by Mansano et al. (2016): Agilent Zorbax ODS C18 column (250 mm × 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. Based on absorbance signals observed in the DAD spectrum of the standard solutions, diuron and carbofuran were detected and quantified at 254 nm and 280 nm, respectively. Analyses were carried out in three replicates. The retention times found for carbofuran and diuron were 3.548 and 4.111 min, respectively. The carbofuran test solutions were analyzed by direct injection in HPLC-DAD, while those of diuron 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 of acetonitrile followed by 10 mL of Milli-Q water, then 50 mL sample was passed through the cartridges under vacuum. The diuron analyte was eluted with 5 mL of acetonitrile, and then subjected to evaporation with nitrogen and reconstituted in 0.5 mL of acetonitrile for chromatographic analysis. This procedure was conducted in triplicate for each diuron test concentration. The detection and quantitation limits, linear correlation coefficients of the curve, precision, accuracy and recovery for the diuron and carbofuran analyses are shown in Supplementary material Table S1.

### 2.3. Single toxicity tests

Toxicity tests with *R. subcapitata* followed the USEPA guideline (USEPA, 2002). After preliminary tests, the concentrations range for each compound was established and toxicity tests were carried out at the following nominal concentration ranges: 1.25–40 µg $L^{-1}$ of diuron and 400–25,600 µg $L^{-1}$ of carbofuran. The nominal concentrations tested for the active ingredients and their commercial formulations were equal.

The assays were performed in 250-mL glass Erlenmeyer flasks containing 100 mL of test solution or LC Oligo medium (control). In the case of diuron, it was necessary to add a solvent control (LC Oligo medium + acetonitrile) with a nominal concentration of 0.04% acetonitrile (v/v). Test flasks were inoculated with an initial concentration of $10^4$ cells $mL^{-1}$ and maintained under the same conditions described for the algal culture. Three replicates were used per treatment. Cells were exposed to the pesticides for 96 h and sampled daily (24, 48, 72 and 96 h) from each flask after manual agitation. The cell counts were carried out by flow cytometry. For each treatment, relative growth rate (RGR) was calculated using the equation described in Bao et al. (2011): $RGR = (N_t–N_0)_{Treatment} / (N_t–N_0)_{Control}$, where $N_t$: the cell density at time t; $N_0$: the initial cell density; and t: exposure duration. Growth inhibition percentage was calculated by comparison of the population growth rates of controls (considered 100%) with the different treatments.

Toxicity tests with the reference substance sodium chloride (NaCl) were performed to evaluate the physiological conditions of the organ-

isms and hence the validity of the tests. Furthermore, the variables pH and temperature of the test solutions were measured at the start and end of the toxicity tests. A preliminary toxicity test comparing the algal cell counts by flow cytometry and direct count using a hemocytometer and optical microscopy was performed using the reference substance NaCl, in order to confirm the accuracy of the method employed.

### 2.4. Analysis by flow cytometry

For algal cell counting, aliquots of control and different treatments of toxicity tests were collected in cryotubes and immediately fixed with formaldehyde buffered with borax (final concentration 1%). The samples were left in the dark for 10 min at room temperature and flash-frozen in liquid nitrogen and stored at −80 °C until analysis. The *R. subcapitata* cells were counted in a FACSCalibur flow cytometer (Becton & Dickinson Franklin Lakes, NJ, U.S.A.) equipped with a 15 mW Argon-ion laser (488 nm emission). For analysis, in 500 µL of subsample were added fluorescent beads (6 µm, Fluoresbrite® carboxylate microspheres, Polysciences Inc., Warrington, PA, U.S.A.) as internal standard. Algal cells were easily identified in cytograms using 90°-side scatter (SSC-H) versus red fluorescence (FL3-H) according to the procedures described in Sarmento et al. (2008). Data acquisition was performed with the BD CellQuest Pro 6 software and analysis of cytograms with the FlowJo v.10.0.8 software. Mean values of FL3-H (chlorophyll *a* fluorescence), SSC-H (cell complexity) and FSC-H (cell size) of the algae and beads population were also extracted and used for the calculation of the relative FL3-H ($FL3\text{-}H_{algae}/FL3\text{-}H_{beads}$), relative SSC-H ($SSC\text{-}H_{algae}/SSC\text{-}H_{beads}$) and relative FSC-H ($SSC\text{-}H_{algae}/SSC\text{-}H_{beads}$), expressed in arbitrary units.

### 2.5. Mixture toxicity tests

After toxicity tests with single pesticides revealed that the effects of active ingredients and their commercial formulations were similar, we chose to perform mixtures tests only with the active ingredients. For toxicity tests of diuron and carbofuran mixtures, an experimental design that included simultaneously both a single test of each pesticide and a set of 23 combinations was chosen for the mixture assay. A partial fixed-ratio design (Cassee et al., 1998) was used for the mixtures tests in order to avoid the inclusion of treatments with the higher concentrations of both pesticides that could lead to mortality of algal cells. Concentrations of the mixtures were based on the expected toxic strengths of 0.375 (0.125 + 0.25; 0.25 + 0.125), 0.5 (0.125 + 0.375; 0.25 + 0.25; 0.375 + 0.125), 0.75 (0.125 + 0.625; 0.25 + 0.5; 0.375 + 0.375; 0.5 + 0.25; 0.625 + 0.125), 1 (0.125 + 0.875; 0.25 + 0.75; 0.375 + 0.625; 0.5 + 0.5; 0.625 + 0.375; 0.75 + 0.25; 0.875 + 0.125), 1.5 (0.5 + 1; 0.75 + 0.75; 1 + 0.5), 1.75 (0.75 + 1; 1 + 0.75) and 2 (1 + 1) toxic units (TU) (Freitas et al., 2014; Pérez et al., 2011). One TU was equal to the $IC_{50}$-96 h obtained from assays with single exposure to each pesticide. The mixture toxicity tests were conducted according to the same protocols used in the single toxicity tests, but with two replicates per treatment. The endpoint evaluated in the mixture assays was the effect on algae population growth rate.

### 2.6. Data analysis

The $IC_{50}$-96 h values of toxicity tests and their respective slope values for single exposures to pesticides were calculated by nonlinear regression, using the three parameter logistic curve (Systat, 2008). This curve is described by the following equation: $Y_i = \max/1 + (C_i/IC50_i)^{\beta i}$; where $Y_i$ is the response of a given parameter; max is its maximum response; $C_i$ is the concentration of chemical *i*; $IC50_i$ is the inhibition concentration of chemical *i* and $\beta i$ is the slope for chemical *i*. The NOEC and LOEC values for tests were obtained by one-way analysis of variance (ANOVA). A post hoc multiple comparisons Dunnett's test was carried out to verify significant differences between treatments and control. In statistical tests, the difference was considered significant when $p \leq 0.05$. All statistical analyses were performed using SigmaPlot version 11.0 software (Systat, 2008).

Data from the mixture toxicity tests were analyzed by comparing the observed data with the expected combined effects from both CA and IA reference models (see model equations in Supplementary material) using the MIXTOX tool (Jonker et al., 2005). In a second step of the data analysis, both CA and IA models were extended as described by Jonker et al. (2005) and deviation functions, such as synergistic/antagonistic interactions, dose-ratio and dose-level dependent deviations were modeled by the addition of two parameters ("a" and "b"), forming a nested framework. In the synergism/antagonism deviation, the parameter "a" becomes, respectively, negative or positive. For dose-ratio dependent deviation (DR), the value of the parameter "$b_{DR}$" in addition to the "a" parameter, indicates that the deviation from the reference model is controlled by the composition of the mixture. For dose-level dependent deviation (DL), the parameter "$b_{DL}$" is included in addition to "a". In this deviation function, the value of "a" indicates the deviation at low doses (i.e., $a > 0$ = antagonism, and $a < 0$ = synergism) and the value of "$b_{DL}$" indicates at what dose level the deviation changes. For more details on these deviation functions please refer Jonker et al. (2005) and Table S2 in Supplementary material. Data were fitted to conceptual models and deviations, and the best fit was chosen by the maximum likelihood method. Where a statistically more descriptive deviation model was identified, the effect pattern was deduced directly from the parameter values (see Table S2 in Supplementary material) and the maximum deviation was calculated in terms of effect level (Freitas et al., 2014; Jonker et al., 2005).

## 3. Results

### 3.1. Abiotic variables of the toxicity tests and chemical analysis

During toxicity tests, the pH values of test solutions remained within the range of 7.4 and 8.2 and did not vary by more than 1.0 unit in any given test. The temperature in all toxicity tests varied between 24.7 and 25.6 °C. Thus, all tests met the validity criteria set forth in the USEPA guidelines (USEPA, 2002).

After analyzing the test solutions in HPLC-DAD, our results showed that, both in the single toxicity tests (active ingredients and commercial formulations) (Supplementary material Fig. S1) and in the mixture toxicity tests of active ingredients (Supplementary material Table S3), the actual exposure concentrations of diuron and carbofuran differed by less than 10% of the nominal concentrations. Therefore, the results were calculated based on nominal concentrations, as suggested by ISO 10706 (2000).

### 3.2. Single toxicity tests

For all compounds tested, the coefficients of variation in replicates of controls of population growth tests did not exceed 10%, as recommended by the USEPA guidelines (2002). In all experiments with active ingredient diuron, control and control solvent had no significant differences ($p > 0.05$), excluding the possibility of solvent to have caused toxic effects on *R. subcapitata*. The reference tests using NaCl indicated that the sensitivity of *R. subcapitata* ($EC_{50}$-48 h = 2.80 ± 0.45 g $L^{-1}$) was within the expected range (reference range: 1.74 a 4.49 g $L^{-1}$) after 96 h exposure.

The algal cell counts by flow cytometry used in toxicity tests may be considered similar to direct counting technique using optical microscopy. These two methods showed a significant correlation ($r = 0.999$, $p < 0.001$, Pearson correlation) and $r^2$ of 0.998 for linear regression of the data (raw data in Supplementary material Table S4).

Population growth curves (cells $mL^{-1}$) for *R. subcapitata* exposed to pesticides diuron and carbofuran (active ingredients and commercial formulations) for 96 h had a strong decrease in cell abundance in the

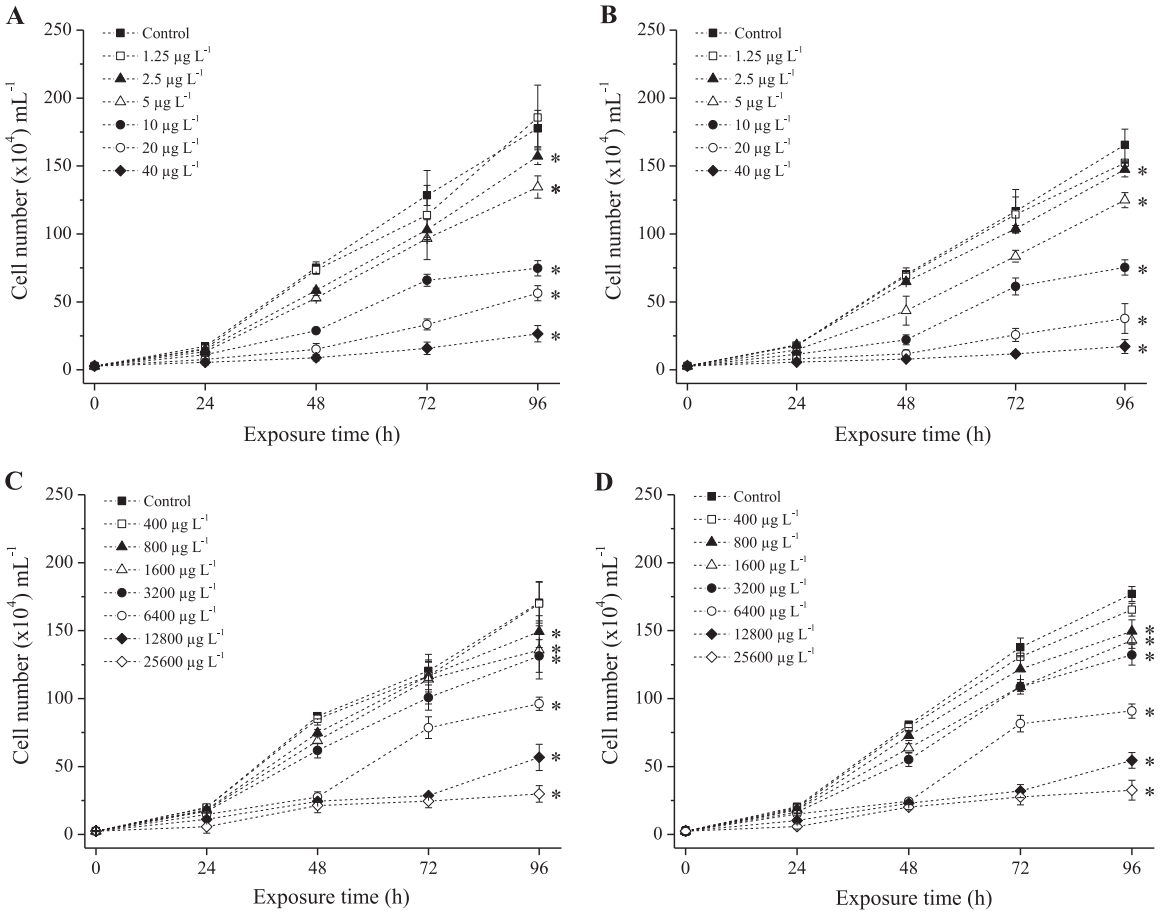


**Fig. 1.** Population growth curves of *Raphidocelis subcapitata* (cell number $\times 10^4$ mL$^{-1}$) after 96 h exposure to different concentrations of diuron (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran (C) and carbofuran dosed as Furadan® 350 SC (D). Asterisk (*) indicates value significantly different from control ($p \leq 0.05$).

highest concentrations tested, indicating a typical concentration-dependent effect (Fig. 1). Compared to control, reductions in algal cell densities were more than 82% at the highest concentrations of compounds after 96 h exposure.

The $IC_{50}$-96 h mean values obtained for each compound in toxicity tests were: 10.4 µg L$^{-1}$ (95% CI: 8.5 − 12.2 µg L$^{-1}$) for active ingredient diuron; 9.3 µg L$^{-1}$ (95% CI: 8.1 − 10.5 µg L$^{-1}$) for diuron dosed as Diuron Nortox® 500 SC; 7426.5 µg L$^{-1}$ (95% CI: 5618.9 − 9234.2 µg L$^{-1}$) for active ingredient carbofuran; and 6974.7 µg L$^{-1}$ (95% CI: 5810.4 − 8139.1 µg L$^{-1}$) for carbofuran dosed as Furadan® 350 SC. For both diuron and carbofuran, the toxicity of the active ingredient for *R. subcapitata* species was similar to its commercial formulation, with no significant difference between them ($p > 0.05$). Furthermore, as expected, the herbicide diuron was more toxic to microalgae *R. subcapitata* than the insecticide carbofuran.

Toxicity tests (96 h) indicated that all compounds tested significantly inhibited the population growth rate of microalgae *R. subcapitata* from a given concentration of the chemical (Fig. 2). For diuron, both active ingredient (Fig. 2A) and commercial formulation (Fig. 2B) significantly inhibited the algal growth in concentrations of 5–40 µg L$^{-1}$, as evidenced by Dunnett's test (active ingredient diuron: $F_{7,16} = 152.4$, $p < 0.001$; commercial formulation diuron: $F_{6,14} = 187.4$, $p < 0.001$). Regarding the growth inhibition, the NOEC of diuron (active ingredient and commercial formulation) was 2.5 µg L$^{-1}$, while the LOEC was 5 µg L$^{-1}$. For carbofuran, significant growth inhibition was observed in algae populations exposed to concentrations of 1600–25,600 µg L$^{-1}$ of carbofuran (Fig. 2C) and 800–25,600 µg L$^{-1}$ of carbofuran dosed as Furadan® 350 SC (Fig. 2D), as evidenced by Dunnett's test (active ingredient carbofuran: $F_{7,16} = 46.8$, $p < 0.001$; commercial formulation carbofuran: $F_{7,16} = 132.4$, $p < 0.001$). The NOEC and LOEC values for active ingredient carbofuran were 800 µg L$^{-1}$ and 1600 µg L$^{-1}$, respectively, while the NOEC and LOEC for carbofuran product were 400 µg L$^{-1}$ and 800 µg L$^{-1}$, respectively.

From the flow cytometry data, significant changes in chlorophyll *a* content (relative FL3-H), cell complexity (relative SSC-H) and cell size (relative FSC-H) of the algae were observed in comparison with the controls (Fig. 3). Diuron, both active ingredient and commercial formulation (Figs. 3A and 3B, respectively), caused a significant increase in relative chlorophyll *a* content of the algae in concentrations of 10–40 µg L$^{-1}$ and also in complexity and cell size in concentrations of 5–40 µg L$^{-1}$. Carbofuran, both active ingredient and commercial formulation (Figs. 3C and 3D, respectively), showed similar effects on algae, significantly increasing the relative chlorophyll *a* content (3200–25,600 µg L$^{-1}$) and the complexity and cell size of algae (12,800 and 25,600 µg L$^{-1}$).

### 3.3. Mixture toxicity tests

The $IC_{50}$-96 h value for each compound tested alone during the mixtures tests was 13.1 µg L$^{-1}$ (95% CI: 11.6 − 14.6 µg L$^{-1}$) for diuron and 8333.2 ± 1898.8 µg L$^{-1}$ (95% CI: 6434.4 − 10,232.0 µg L$^{-1}$) for carbofuran. Although the mode of action of diuron (phenylurea) is well known for algae, i.e., it is a potent inhibitor of photosynthesis, the mode of action of carbofuran (carbamate) on the algae is not yet well established. Therefore, in this study, two reference models (CA and IA) were tested to assess the *R. subcapitata* response when exposed to mixtures of these two pesticides.

All parameters and significance test results obtained by fitting the nested MIXTOX tool are shown in Table 1. The fitting of the mixture data to the CA model yielded a sum of squared residuals (SS) of 0.62

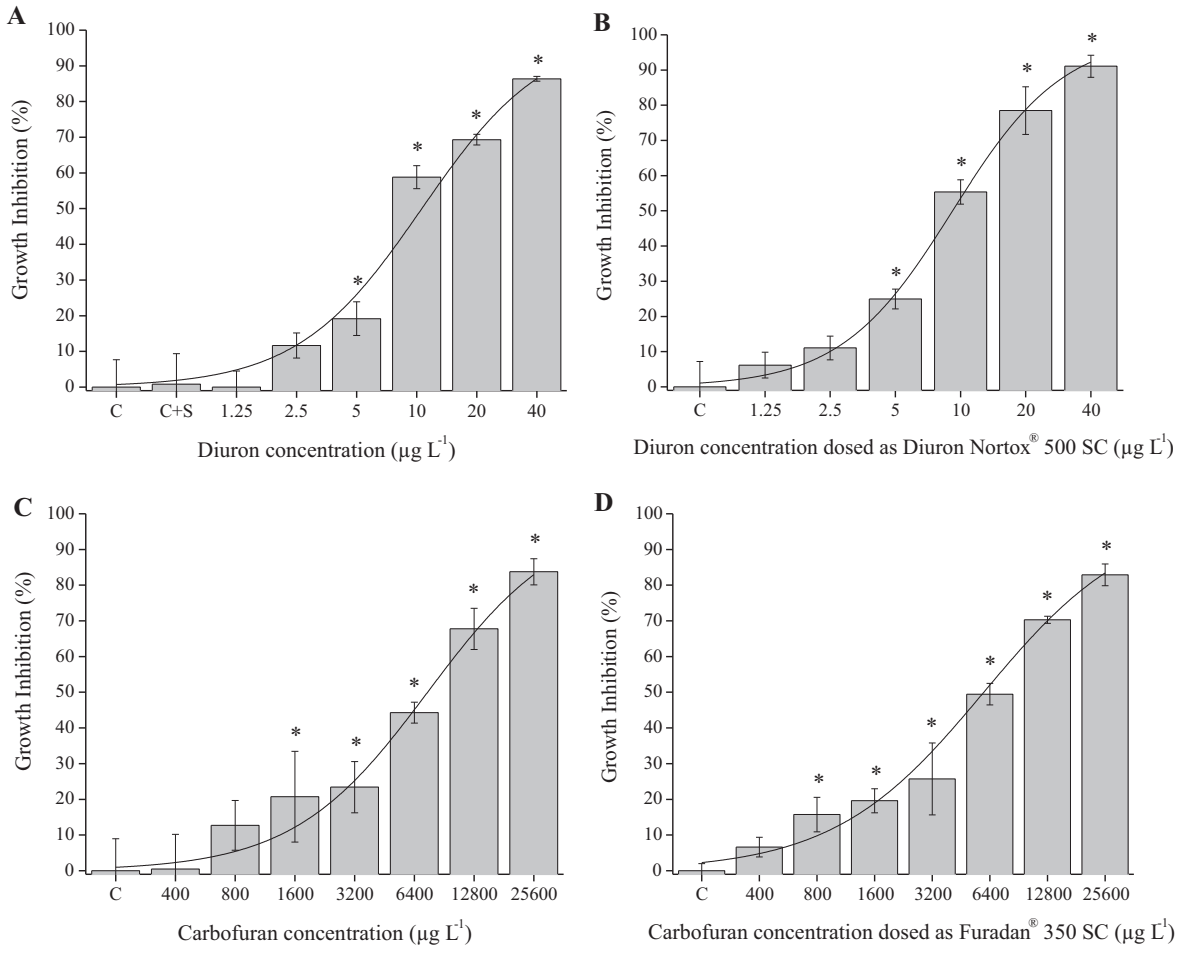


**Fig. 2.** Inhibition of population growth rate (%) of *Raphidocelis subcapitata* exposed to different concentrations of diuron (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran (C) and carbofuran dosed as Furadan® 350 SC (D). The line represents the logistic curve-fitting. Asterisk (*) indicates value significantly different from control ($p \leq 0.05$).

($p < 0.05$; $r^2 = 0.81$). After adding parameter "a" to the model in order to describe the S/A deviation, the SS value decreased to 0.39 and was statistically significant ($p < 0.05$; $r^2 = 0.88$). For dose-ratio dependent (DR) deviation, when the parameters "a" and "$b_{DR}$" were added, there was a decrease of the SS value to 0.32, which was statistically significant ($p < 0.05$; $r^2 = 0.90$). The dose-level dependent (DL) deviation was not significant ($p = 0.854$) (Table 1). Thus, DR deviation from the CA model presented the best fit, and explained 90% of the variation of data set for the model. Therewith, it was verified that the interaction of the pesticides in the mixtures was dose-ratio dependent (DR), being that antagonism at high carbofuran concentrations and low diuron concentrations and synergism at high diuron concentrations and low carbofuran concentrations occurred (Fig. 4A). The synergism observed in the mixtures was mainly caused by diuron.

The fitting of the mixture data to the IA model yielded a SS value of 0.54 ($p < 0.05$; $r^2 = 0.83$). After adding parameter "a" to the model to describe the S/A deviation, the SS value decreased to 0.37 and was statistically significant ($p < 0.05$; $r^2 = 0.88$). DR and DL deviations were not statistically significant ($p = 0.209$ and $p = 0.212$, respectively) (Table 1). Therefore, S/A deviation from the IA model presented the best fit for the data and indicated synergistic interactions (increased toxicity) between diuron and carbofuran when in mixtures (Fig. 4B). Although the DR deviation from the IA was not statistically significant, Fig. 4B shows that synergism occurred when diuron was the dominant chemical in mixtures, with the possibility of antagonism occurrence when carbofuran is in high concentrations in the mixtures.

## 4. Discussion

### 4.1. Toxicity of single pesticides

In this study, the toxicity of the active ingredients diuron and carbofuran to *R. subcapitata* had no significant difference with that of their commercial products (Diuron Nortox® 500 SC and Furadan® 350 SC, respectively). Although most studies report a higher toxicity of formulated product when compared to active ingredient for different species and commercial formulations (e.g. Beggel et al., 2010; Kroon et al., 2015; Mullin, 2015; Pereira et al., 2009), different responses are expected to occur due to the associated inert ingredients and toxicity responses be usually species-specific. Pessoa et al. (2011) comparing the toxicity of carbofuran and the same commercial product (Furadan® 350 SC) for *Oreochromis niloticus* fish larvae found similar $LC_{50}$-96 h values for these compounds, indicating that the toxic potential of the active ingredient was not affected by the inert substances added to the commercial formulation, as occurred in our study.

As expected, the herbicide diuron was highly toxic to microalgae *R. subcapitata*, while the insecticide/acaricide/nematicide carbofuran was only slightly toxic to this species. The high toxicity of diuron on algae and aquatic plants is due to the specific mode of action of this compound on autotrophic organisms. This herbicide binds to the plastoquinone site (QB) on D1 protein blocking the electron transfer in Photosystem II and thus inhibiting the process of photosynthesis (Krieger-Liszkay, 2005). On the other hand, the carbofuran is an acetylcholinesterase inhibitor, considered very toxic to fish and aquatic invertebrates (Ibrahim and Harabawy, 2014), but for autotrophic

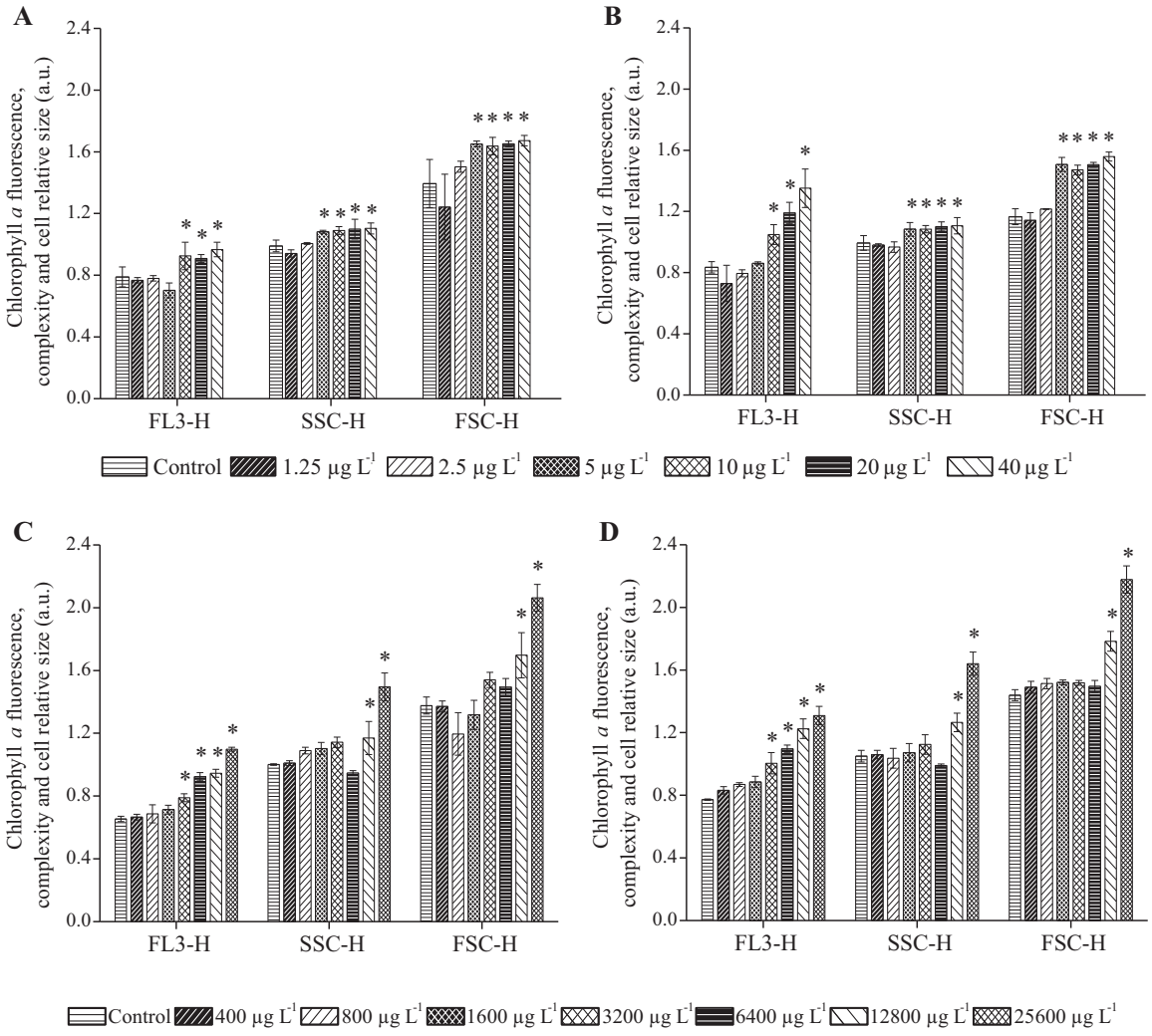


**Fig. 3.** Mean values of chlorophyll *a* fluorescence (relative FL3-H), complexity (relative SSC-H) and cell size (relative FSC-H) of the population of *Raphidocelis subcapitata* exposed to different concentrations of diuron (A), diuron dosed as Diuron Nortox® 500 SC (B), carbofuran (C) and carbofuran dosed as Furadan® 350 SC (D). Asterisk (*) indicates value significantly different from control ($p \leq 0.05$). Values are expressed in arbitrary units (a.u.).

**Table 1.**
Parameters and fit tests of the reference models concentration addition and independent action applied to population growth rate of *Raphidocelis subcapitata* exposed for 96 h to mixtures of diuron and carbofuran.

| | **Concentration addition** | | | | **Independent action** | | | |
|---|---|---|---|---|---|---|---|---|
| | **CA** | **S/A** | **DR** | **DL** | **IA** | **S/A** | **DR** | **DL** |
| Max | 0.93 | 0.92 | 0.92 | 0.92 | 0.93 | 0.93 | 0.93 | 0.93 |
| $\beta_{Diuron}$ | 2.13 | 2.90 | 2.79 | 3.40 | 3.22 | 4.04 | 4.16 | 2.87 |
| $\beta_{Carbofuran}$ | 7.32 | 705.85 | 705.85 | 705.86 | 7.84 | 19.07 | 65.94 | 17.03 |
| $IC_{50}$ for Diuron | 15.98 | 11.69 | 14.26 | 7126.41 | 9.63 | 13.21 | 12.81 | 14.19 |
| $IC_{50}$ for Carbofuran | 12,786.18 | 12,773.73 | 12,773.73 | 12,773.96 | 10,642.73 | 10,642.78 | 10,643.94 | 10,642.81 |
| a | – | 0.90 | 2.36 | −0.05 | – | −4.50 | −7.66 | −1.83 |
| $b_{DR/DL}$ | – | – | −3.83 | 13.51 | – | – | 5.48 | −4.97 |
| SS | 0.62 | 0.39 | 0.32 | 0.39 | 0.54 | 0.37 | 0.36 | 0.36 |
| $r^2$ | 0.81 | 0.88 | 0.90 | 0.88 | 0.83 | 0.88 | 0.89 | 0.89 |
| $\chi^2$ or F test | 33.84 | 17.21 | 7.33 | 0.03 | 40.05 | 13.60 | 1.58 | 1.56 |
| df | – | 1.00 | 1.00 | 1.00 | – | 1.00 | 1.00 | 1.00 |
| $p$ ($\chi^2$/F) | $4.45 \times 10^{-11}$ | 0.00003 | 0.007 | 0.854 | $5.00 \times 10^{-12}$ | 0.0002 | 0.209 | 0.212 |

Max is the maximum response value; $\beta$ is the slope of the individual dose response curve; $IC_{50}$ is the median growth inhibition concentration; a, $b_{DR}$ and $b_{DL}$ are parameters of the function; SS is the sum of squared residuals; $r^2$ is the regression coefficient; $\chi^2$ or F test is the test statistic; df is the degrees of freedom; and $p$ ($\chi^2$ / F) is the significance level of the test statistic. CA is concentration addition model and IA is independent action model, S/A is synergism or antagonism deviation, DR is dose-ratio dependent deviation and DL is dose-level deviation.

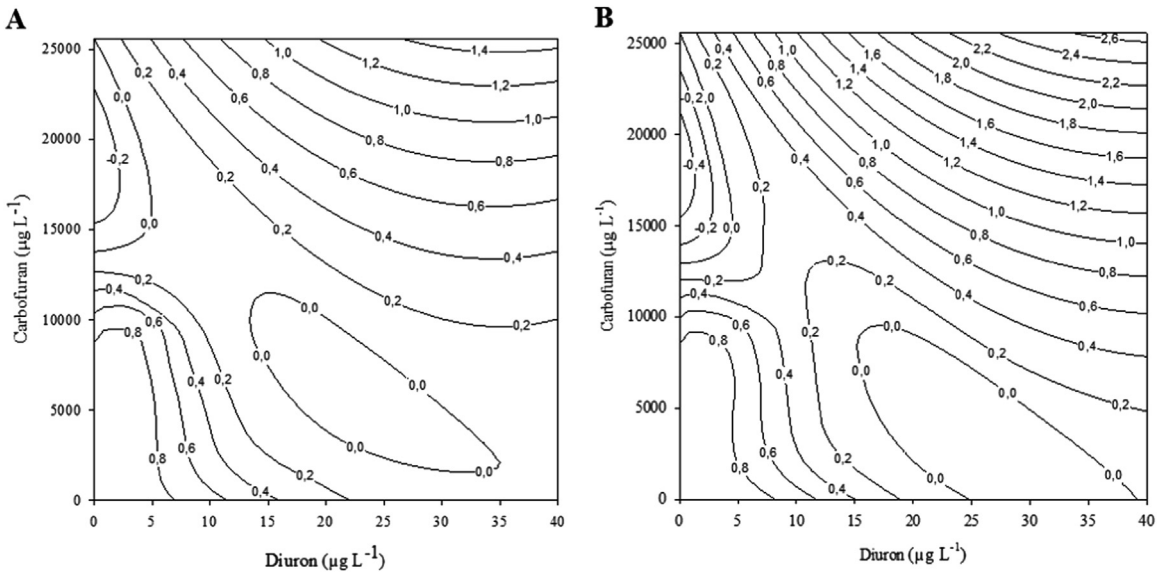


**Fig. 4.** Isobolograms of the pesticide mixture effects on population growth rate of *Raphidocelis subcapitata*. (A) dose-ratio dependent deviation (DR) from the concentration addition model (CA) and (B) synergism or antagonism deviation (S/A) from the independent action model (IA).

organisms its mode of action is still unclear.

Exposure to diuron and carbofuran significantly decreased the algae density and growth rate compared to the control at the highest concentrations of these compounds. These data are consistent with those of other authors, which reported effects of these pesticides on microalgae cell growth (e.g. Arzul et al., 2006; Ma et al., 2006a, 2006b; Zhang et al., 2012). Ma et al. (2006b) evaluated the toxicity of 40 herbicides on algae *R. subcapitata* and considering the parameter growth inhibition, the herbicide diuron was the most toxic one. Reduction in density and growth rate may alter the composition of planktonic and benthic algal communities. Several studies have shown negative effects of diuron exposure on biomass (as chlorophyll *a*) and primary production in phytoplankton communities (Knauer et al., 2010; Knauert et al., 2008, 2009; Perschbacher and Ludwig, 2004) and periphyton (López-Doval et al., 2010; Ricart et al., 2009; Tlili et al., 2008, 2010).

The $IC_{50}$-96 h value for *R. subcapitata* exposed to diuron (active ingredient) in this study was similar to that verified by Fai et al. (2007), lower than that registered by Zhang et al. (2012) and higher than the value observed by Ma et al. (2006b) (Table 2). Compared with other autotrophic organisms, *R. subcapitata* was more sensitive to diuron than the cyanobacterium *Synechococcus* sp., the algae *Achnanthidium minutissimum*, *Chaetoceros gracilis*, *Craticula accomoda*, *Desmodesmus subspicatus* and *Navicula forcipata* and the macrophyte *Lemna minor* (see Table 2).

Diuron also caused physiological (cell chlorophyll *a* content) and morphological (cell complexity and size) changes in *R. subcapitata* cells. After 96 h exposure, it was verified that diuron significantly increased the relative chlorophyll *a* content as well as the complexity and size of algae cells, compared with control. Similar effects on chlorophyll *a* content have been observed in other studies where microalgae were exposed to photosynthesis inhibitors such as atrazine (Adler et al., 2007), isoproturon and terbutryn (Rioboo et al., 2002), and also diuron (Magnusson et al., 2008; Stachowski-Haberkorn et al., 2013). Several studies suggested that this effect could be an adaptive strategy to compensate the herbicide action (e.g. Magnusson et al., 2008; Ricart et al., 2009). As the diuron inhibits photosynthesis, cells may produce more chlorophyll *a* in order to maximize light harvesting. Regarding the cell relative complexity and size, Stachowski-Haberkorn et al. (2013) observed a significant decrease in these parameters when cells of microalgae *Tetraselmis suecica* were exposed to diuron, contrasting to the results observed in this study for *R. subcapitata*. A possible explanation for cell size increase may be related to uncoupling of cell growth and cell division (i.e., the incapacity to finish cell division) (Jamers and De Coen, 2010).

**Table 2.**
Values of toxicity ($EC_{50}$ or $IC_{50}$) obtained from the literature for different autotrophic species exposed to pesticides diuron and carbofuran.

| | Endpoint | Value (µg $L^{-1}$) | Reference |
|---|---|---|---|
| **Diuron** | | | |
| *Achnanthidium minutissimum* | $IC_{50}$ 96 h | 108.0 | Larras et al. (2012) |
| *Chaetoceros gracilis* | $IC_{50}$ 72 h | 36.0 | Koutsaftis and Aoyama (2006) |
| *Chlorella pyrenoidosa* | $IC_{50}$ 96 h | 2.3 | Ma et al. (2002b) |
| *Chlorella vulgaris* | $IC_{50}$ 96 h | 4.3 | Ma et al. (2002a) |
| *Craticula accomoda* | $IC_{50}$ 96 h | 1734.0 | Larras et al. (2012) |
| *Desmodesmus subspicatus* | $IC_{50}$ 72 h | 46.3 | Masojídek et al. (2011) |
| *Dunaliella tertiolecta* | $EC_{50}$ 96 h | 9.2 | DeLorenzo et al. (2013) |
| *Lemna minor* | $IC_{50}$ 168 h | 28.3 | Gatidou et al. (2015) |
| *Navicula forcipata* | $IC_{50}$ 96 h | 27.0 | Gatidou and Thomaidis (2007) |
| *Nephroselmis pyriformis* | $IC_{50}$ 72 h | 4.7 | Magnusson et al. (2008) |
| *Raphidocelis subcapitata* | $IC_{50}$ 96 h | 10.4 | Present study |
| *Raphidocelis subcapitata* | $IC_{50}$ 72 h | 10.5 | Fai et al. (2007) |
| *Raphidocelis subcapitata* | $IC_{50}$ 96 h | 0.4 | Ma et al. (2006b) |
| *Raphidocelis subcapitata* | $IC_{50}$ 72 h | 22.3 | Zhang et al. (2012) |
| *Scenedesmus obliquus* | $IC_{50}$ 96 h | 4.1 | Ma (2002) |
| *Scenedesmus quadricauda* | $IC_{50}$ 96 h | 2.7 | Ma et al. (2003) |
| *Skeletonema costatum* | $IC_{50}$ 96 h | 5.9 | Bao et al. (2011) |
| *Synechococcus* sp. | $EC_{50}$ 96 h | 110.0 | Bao et al. (2011) |
| *Thalassiosira pseudonana* | $IC_{50}$ 96 h | 4.3 | Bao et al. (2011) |
| **Carbofuran** | | | |
| *Anabaena flos-aquae* | $IC_{50}$ 96 h | 7926.3 | Ma et al. (2006a) |
| *Chaetoceros gracilis* | $EC_{50}$ 72 h | 5110.0 | Arzul et al. (2006) |
| *Chlorella pyrenoidosa* | $IC_{50}$ 96 h | 14,633.3 | Ma et al. (2006a) |
| *Chlorella vulgaris* | $IC_{50}$ 96 h | 7864.6 | Ma et al. (2006a) |
| *Chlorella vulgaris* | $EC_{50}$ 72 h | 9960.0 | Arzul et al. (2006) |
| *Microcystis aeruginosa* | $IC_{50}$ 96 h | 4649.7 | Ma et al. (2006a) |
| *Microcystis flosaquae* | $IC_{50}$ 96 h | 11,260.5 | Ma et al. (2006a) |
| *Phaeodactylum tricornutum* | $EC_{50}$ 72 h | 7130.0 | Arzul et al. (2006) |
| *Raphidocelis subcapitata* | $IC_{50}$ 96 h | 7426.5 | Present study |
| *Raphidocelis subcapitata* | $EC_{50}$ 72 h | 2600.0 | Iesce et al. (2006) |
| *Raphidocelis subcapitata* | $IC_{50}$ 72 h | 158.2 | Dobsikova (2003) |
| *Raphidocelis subcapitata* | $IC_{50}$ 96 h | 6219.1 | Ma et al. (2006a) |
| *Scenedesmus acutus* | $IC_{50}$ 96 h | 6774.5 | Ma et al. (2006a) |
| *Scenedesmus quadricauda* | $IC_{50}$ 96 h | 37,875.6 | Ma et al. (2006a) |

For carbofuran, previous ecotoxicological studies (e.g. Arzul et al., 2006; Dobsikova, 2003; Iesce et al., 2006; Ma et al., 2006a) showed adverse effects of this insecticide on non-target algal primary producers. According to Peterson et al. (1994), carbofuran was highly toxic to green microalgae *Scenedesmus quadricauda* and moderately toxic to cyanobacterium *Mycrocystis aeruginos*a at expected environmental concentration of 667 μg $L^{-1}$. Arzul et al. (2006), when evaluating the population growth rate of algae exposed to carbofuran found that this pesticide caused significant hormetic effects on *Chlorella vulgaris*, wherein the concentration of 880 μg $L^{-1}$ of carbofuran stimulated the growth and concentrations from 4620 μg $L^{-1}$ of carbofuran reduced the growth rate of this alga. Moreover, these same authors observed significant growth inhibition of marine algae *Chaetoceros gracilis* and *Phaeodactylum tricornutum* exposed to carbofuran.

In our study, the $IC_{50}$-96 h value for *R. subcapitata* exposed to carbofuran (active ingredient) was similar to that recorded by Ma et al. (2006a), but higher than the values observed by Dobsikova (2003) and Iesce et al. (2006) (Table 2). Compared with other autotrophic organisms, *R. subcapitata* was more sensitive to carbofuran than the cyanobacterium *Microcystis flosaquae* and the algae *Chlorella pyrenoidosa*, *C. vulgaris* and *Scenedesmus quadricauda* (see Table 2).

As observed for diuron, carbofuran also significantly increased the chlorophyll *a* content, complexity and cell size of *R. subcapitata*, when compared with control. Many pesticides may interfere with cell growth and division by preventing normal mitotic processes from occurring (DeLorenzo et al., 2001) beside the accumulation of macromolecules with subsequent cell size increase (Kent and Currie, 1995). In the study of Megharaj et al. (1993), the chlorophyll *a* concentration of *Chlorella vulgaris* increased at carbofuran concentrations of 2 and 5 mg $L^{-1}$ and decreased above 10 mg $L^{-1}$. According to these authors, photosynthetic apparatus of this alga was greatly disturbed in cells grown in the presence of carbofuran. In this study, carbofuran probably reduced the algae cell divisions in the highest concentrations tested, the cells remained larger and therefore cell size and chlorophyll *a* content increased. Moreover, Azizullah et al. (2011) reported that carbofuran may affect the cell size and shape by osmotic stress or interaction with cell plasma membrane.

Diuron and carbofuran have been found at high concentrations in water bodies worldwide (e.g. Faggiano et al., 2010; Kaonga et al., 2015; Masiá et al., 2015; Papadakis et al., 2015). In Brazil, maximum reported concentrations range from 0.9 to 408 μg $L^{-1}$ for diuron (e.g. Britto et al., 2012; Dantas et al., 2011; Dores et al., 2009; Paschoalato et al., 2008) and from 0.1 to 68.8 μg $L^{-1}$ for carbofuran (e.g. Caldas et al., 2011; Carbo et al., 2008; Loro et al., 2015). According to these environmental concentrations, diuron doses used in this study are ecologically relevant and our results demonstrate that this herbicide presents high ecological risk of causing toxic effects on algae in Brazilian water bodies, and may alter species composition, community structure and functioning of aquatic ecosystems. In the case of carbofuran, if this compound occurred alone in the environment, autotrophic organisms would be apparently not affected at these actual concentrations based on the toxicity values obtained in the present study. However, environmental concentrations observed for carbofuran may cause toxic effects on sensitive primary consumers (e.g. microcrustaceans and fish larvae) and indirectly affect microalgae by reduction of their herbivore predators.

### 4.2. Toxicity of pesticide mixtures

In this study, data of mixtures toxicity tests fitted well in both reference models (CA and IA), but showed different deviations. For CA model, dose-ratio dependent (DR) deviation presented the best fit and its parameters indicated antagonism at high carbofuran concentrations and low diuron concentrations and synergism at high diuron concentrations and low carbofuran concentrations, being that the synergism of toxic mixture was mainly caused by diuron. For IA model, synergism/antagonism (S/A) deviation showed the best fit to data and indicated synergistic interactions between diuron and carbofuran when combined. In general, both models evidenced the occurrence of synergism in the mixtures of these compounds, especially when diuron was the dominant chemical in the mixture. Moreover, considering that the diuron concentrations tested are environmentally relevant and only low concentrations of carbofuran may occur in the environment, the effect of synergism in the mixtures of these compounds is most likely to occur in aquatic ecosystems.

According to literature data, this is the first study that evaluated the effects of mixtures of diuron and carbofuran on microalgae *R. subcapitata*. For this species, so far, studies of mixtures were performed using other pesticides. As example may be cited the researches of Pérez et al. (2011), which analyzed the effects of binary mixtures of herbicides (atrazine, simazine, terbuthylazine and metolachlor) on the growth rate of *R. subcapitata* and of Fernández-Alba et al. (2002), which evaluated the effects of binary combinations of herbicides (diuron, irgarol, tributyltin and Kathon 5287) and fungicides (chlorothalonil, dichlofluanid and TCMTB) on *R. subcapitata* (named as *Selenastrum capricornotum*). Binary mixtures of herbicides (diuron and irgarol) or an herbicide with a fungicide (irgarol and chlorothalonil; irgarol and TCMTB) resulted in synergistic interactions in the study Fernández-Alba et al. (2002).

DeLorenzo and Serrano (2003) analyzed the toxicity of pesticide mixtures (atrazine and chlorpyrifos; atrazine and chlorothalonil) for marine alga *Dunaliella tertiolecta* (Chlorophyta). The combination of atrazine (herbicide) and chlorpyrifos (insecticide) exhibited an additive toxicity, while the mixture of atrazine and chlorothalonil (fungicide) caused a synergistic effect where the toxicity of mixture was approximately 2 times higher than that of the individual chemicals (DeLorenzo and Serrano, 2003).

In a review by Cedergreen (2014), these authors found that in 95% of 69 cases of synergism described to pesticides, the mixtures synergistic effect include cholinesterase inhibitors and azole fungicides. According to these researchers, both groups of pesticides are known to interfere in the metabolic degradation of other xenobiotics. However, cases of synergy involving a cholinesterase inhibitor in binary mixtures of pesticides for autotrophic organisms have not yet been reported (Cedergreen, 2014). Thus, we emphasize the importance of this study, which presents a case of synergistic interaction of the mixture of a cholinesterase inhibitor (carbofuran) with a phenylurea (diuron) to microalgae *R. subcapitata*.

Regarding the toxicity to organisms, the interactions between chemicals can affect several processes, such as bioavailability, adsorption, distribution, metabolism (biotransformation), binding to target site and excretion. The synergistic interactions are probably caused by interactions linked to one or more of these processes (Cedergreen, 2014). Studies verified that the mixture of a triazine (atrazine) with an organophosphate (chlorpyrifos) caused synergistic interactions in *Chironomus tentans* larvae (Belden and Lydy, 2000) and *Danio rerio* larvae (Pérez et al., 2013). The increased toxicity of these compounds in mixture was explained by the fact atrazine induce the cytochrome P450 enzyme system and increase the chlorpyrifos biotransformation rate, converting it into a more toxic metabolite (Pérez et al., 2013). Cedergreen (2014) proposed that the synergistic interaction between metals and herbicides photosynthesis inhibitors in autotrophic organisms could be due to the interaction of metals with enzymes responsible for repairing, which would prevent the damaged photosystem II repair and also the damage caused by reactive oxygen species (ROS) produced by inhibition of photosynthesis and the metals themselves. Although there are examples of mechanisms that cause synergy in invertebrate species and autotrophic organisms, there is no information in the literature on a similar mechanism that can explain the synergistic interactions of diuron and carbofuran mixture on *R. subcapitata*.

According to Neuwoehner et al. (2010), for *R. subcapitata* the diuron degradation products (3,4-DCA, DCPU, MCPDMU) are less toxic than

the parent compound. Thus, perhaps the increase of diuron biotransformation rates do not explain the increased toxicity to *R. subcapitata*. For future work, research on the mechanisms responsible for synergism between diuron and carbofuran are important.

Studies in Brazil (e.g. Carbo et al., 2008) and elsewhere around the world (Bacigalupo and Meroni, 2007; Faggiano et al., 2010; Masiá et al., 2015) revealed the simultaneous presence of diuron and carbofuran in aquatic environments. According to the results of this study, the mixture of carbofuran and diuron in the aquatic environment may lead to increased toxicity to algae and thus cause more devastating effects on ecosystems. As algae are the base of most food webs, any effect on them will affect the higher trophic levels, which enhances the importance of studying the impact of pesticides toxicity on algae. For a better protection of aquatic ecosystems, it is necessary that the regulatory agencies consider the mixtures toxicity for algae and not only the safe concentrations for individual compounds, since the occurrence of mixtures of several chemicals is common in aquatic environments.

## 5. Conclusions

Results of effects of diuron and carbofuran mixture on algae *R. subcapitata* obtained in this study are unprecedented. According to fitting the toxicity data to the CA and IA reference models, synergistic interactions between these pesticides may occur, especially when diuron is the dominant chemical in the mixture. Synergy cases involving a cholinesterase inhibitor in pesticides binary mixtures to autotrophic organisms have not been reported in the literature until now, which enhances the relevance of this study. For this algal species, the toxicity of active ingredients and their commercial formulations had no significant differences, indicating that the toxic potential of active ingredients were not affected by “inert” substances added to commercial formulations. From the results of single toxicity tests, both pesticides caused adverse toxic effects on microalgae *R. subcapitata*. Environmentally relevant concentrations of diuron significantly inhibit the algae population growth and caused physiological (chlorophyll *a* content) and morphological (complexity and cell size) changes in cells. Despite the toxicity of carbofuran occur at high concentrations, this pesticide in low concentrations can interact with diuron and increase the toxicity to algae. The increased algae toxicity caused by diuron and carbofuran mixture may pose a greater environmental risk for the phytoplankton. Thus, since diuron and carbofuran can be found in mixtures in natural environments, our results reinforce that ecological risk assessments should consider the pesticides mixture toxicity in order to avoid under- or over-estimation of their effects on phytoplankton.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.04.024.

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