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Caffeine reduces the toxicity of albendazole and carbamazepine to the microalgae *Raphidocelis subcapitata* (Sphaeropleales, Chlorophyta)

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Abstract

Pharmaceutically active compounds (PhACs) are emerging contaminants that have been widely detected in water bodies in the last decades, with ecological effects toward aquatic biota that have not been fully elucidated. Most studies concerning their toxicity to microalgae have only considered short-term individual PhAC exposure, rather than combined exposure to several compounds for longer time periods. In this study, we investigated the effects of albendazole (ABZ) (anthelmintic) and carbamazepine (antiepileptic), alone and in combination with caffeine, on the growth and production of chlorophyll-a of the microalgae *Raphidocelis subcapitata*, during 16 days of exposure. ABZ alone had a more significant effect than carbamazepine alone on the growth rate and maximum cell density of the microalgae ($p < .05$; analysis of variance). These results were probably related to the effect of ABZ in inhibiting enzyme complexes and cell membrane proteins related to adenosine triphosphate synthesis, which is important for cell growth. The presence of caffeine lowered the toxicities of ABZ and carbamazepine to the microalgae, probably due to its antioxidant properties, positively affecting chlorophyll-a production, growth rate, and maximum cell density. Thus, caffeine had an antagonistic interaction with the studied PhACs. The results reinforce the importance of ecotoxicological assays that compare individual and combined PhAC exposure conditions. Our findings highlighted that caffeine can be a relevant factor influencing such assays, considering its widespread occurrence in impacted water bodies.

KEYWORDS

chlorophyceae, ecotoxicological assays, emerging contaminants, pharmaceutically active compounds

1 | INTRODUCTION

Many man-made compounds are continuously released into different environmental compartments by anthropogenic activities worldwide (García-García, Sarma, Núñes-Orti, & Nandini, 2014), due to the intensive use of these substances in various industrial and commercial sectors, including health care (Xiong, Kurade, Kim, Roh, & Jeon, 2017). In aquatic systems, the detection of emerging contaminants such as pharmaceutically active compounds (PhACs), personal care products, and industrial wastes has increased in the last few decades (Leal, Mesquita, Amaral, Amaral, & Ferreira, 2020; Peña-Guzmán et al., 2019). Brazilian laws do not fully address these substances (there are no

specific guidelines or legal standards for their permissible concentrations) and very limited information is available about their fate and occurrence in the country.

Emerging contaminants can have profound effects on the characteristics of aquatic communities, increasing the costs of drinking water treatment and decreasing overall water quality in lakes, rivers, and streams, leading to limitations in terms of uses of the water (Bolong, Ismail, Salim, & Matusurra, 2009; Kümmerer, 2008; Petrovic, Gonzalez, & Barceló, 2003). Antibiotic-resistant bacteria growing in PhACs-enriched waters can compromise public and environmental health (Baquero, Martínez, & Cantón, 2008; Horvat et al., 2012; Pruden et al., 2006; Sutherland & Leathwick, 2011). Some compounds can bioaccumulate in aquatic organisms, due to biomagnification in the food chain, which is increased by their characteristics including recalcitrance and lipophilicity (Kelly, Ikonomou, Blair, Morin, & Gobas, 2007). However, the effects on human health of bioaccumulation and biomagnification of PhACs in the food chain are still unclear (Boonsaner & Hawker, 2013).

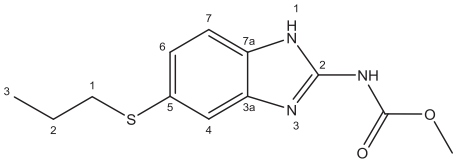
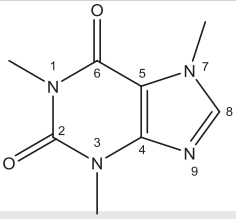
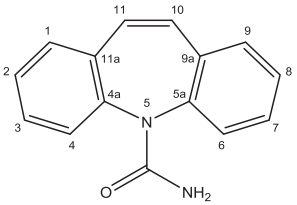
Albendazole (ABZ), an anthelmintic drug used in human and veterinary medicines, can be readily adsorbed in soils, sediments, and the sludge of wastewater treatment plants (WWTPs), due to its hydrophobic properties (Table 1). However, pH shifts can increase the polarity of the molecule (Fagundes, 2018; Jung, Medina, García, Fuentes, & Moreno-Esparza, 1997) and its solubility in water, making ABZ more available in aquatic ecosystems (Liou & Chen, 2018). ABZ and its main metabolite (ricobendazole) were detected in raw wastewater from a hospital in Campinas (São Paulo State, Brazil) at concentrations of up to 3,810 and 3,894 ng/L, respectively. In this case, the WWTP system was able to remove 46–95% of the ABZ, with final concentrations ranging from 16 to 441 ng/L (Porto, Rodrigues-Silva, Schneider, & Rath, 2019).

Carbamazepine (CBZ; Table 1) is an antiepileptic drug with a worldwide per capita consumption of 169 mg/year (Zhang & Geissen, 2008). CBZ has been detected in WWTP effluents at concentrations up to the micrograms per liter level, examples being 2.3 µg/L in Canada (Metcalfe, Miao, Koenig, & Struger, 2003) and 6.3 µg/L in Germany (Ternes, 1998). Although no acute toxicity toward aquatic biota is expected at these levels, the chronic effects remain unknown (Jos et al., 2003; Ternes, 1998) and the predicted no-effect concentration (PNEC) for CBZ is 0.42 µg/L. Although PNEC values are intended to be conservative, this suggests that CBZ could affect the aquatic biota (TGD, 1996), as the occurrence of such PhAC in water bodies is becoming increasingly common (W. Zhang et al., 2012).

Caffeine (CAF; Table 1) is a central nervous system stimulant consumed worldwide, which is frequently found at micrograms per liter levels in aquatic environments (Peteffi et al., 2018). In Brazil, CAF concentrations of 1.6–3.5 and 290 µg/L were reported in streams (Peteffi et al., 2018) and untreated sewage (Canela, Jardim, Sodré, & Grassi, 2014), respectively. CAF presents high chemical stability, has an aquatic half-life between 3 days and 3 months, and is a marker of sewage contamination (Peteffi et al., 2018).

Several studies have already been carried out to evaluate the toxicities of PhACs to microalgae (Miazek & Brozek-Pluska, 2019). In general, the results suggested that PhACs at certain levels (ng/L or µg/L) did not exhibit acute toxicity to microalgae, but information

TABLE 1 Structural formulae of the PhACs studied and some of their physicochemical characteristics

Properties/PhAC	Albendazole	Caffeine	Carbamazepine
Structural formula			
Chemical Abstracts Service (CAS)	54965-21-8	58-08-2	298-46-4
Molar mass (g/mol)	265.3	194.2	236.1
Molar volume (cm^3/mol)	203.1	133.3	186.5
Water solubility (g/L)	0.010	21.700	0.152
Log K_{ow}	2.2–2.92	−0.16	2.45
Log K_{oc}	2.94	1.00	3.10
pK_a	5.54–13.11	0.52–14.00	15.96
Expected action in humans/pathogens	Inhibition of tubulin polymerization	Binding to and blocking of adenosine receptors	Blockage of voltage-sensitive sodium channels

Abbreviation: PhAC, pharmaceutically active compound.

Source: Alygizakis et al. (2016); Barrowman, Marriner, and Bogan (1984); Crane, Watts, and Boucard (2006); Danaher, De Ruyck, Crooks, Dowling, and O'Keeffe (2007); Horvat et al. (2012); SciFinder (2019).

concerning their chronic effects is still limited (Miazek & Brozek-Pluska, 2019; W. Zhang et al., 2012). Most of the reported studies considered short exposure times, such as 24, 72, or 96 hr (Yang et al., 2008; Zounková et al., 2007). However, microalgae are exposed continuously to PhACs, due to the inputs of the compounds into water bodies, especially in developing countries with limited infrastructure related to wastewater collection and treatment. Moreover, most evaluations have focused on the effects of a single PhAC, while aquatic communities are usually exposed to mixtures of PhACs in different proportions (Kümmerer, 2008). A simple binary mixture of PhACs (such as sulfonamides, tylosin, trimethoprim, and tetracyclines) may have additive effects (Liu et al., 2018). This was also observed by Yang et al. (2008) in 72-hr ecotoxicological assays with trimethoprim and sulfonamide, which synergistically influenced the growth rate of *Raphidocelis subcapitata*. The same authors observed that the binary combinations of "tylosin and triclocarban," "triclocarban and norfloxacin," and "triclosan and norfloxacin" had antagonistic effects on the microalgae growth rate, in comparison to the effects of the individual compounds.

The aim of the present study was to investigate the ecotoxicological effects of different PhACs, considering individual and combined exposure treatments (ABZ, CBZ, ABZ + CAF, and CBZ + CAF). Assays were performed using the microalgae *R. subcapitata*, evaluating parameters related to its growth and development under controlled laboratory conditions. The exposure tests were continued for periods longer than typically employed in studies reported in the literature, to be able to observe ecotoxicological effects during all growth phases of the microalgae. The hypothesis adopted was that in the case of mixtures of ABZ or CBZ with CAF, the interaction between the compounds would lead to different types of effects in *R. subcapitata*, especially considering that CAF has antioxidant properties. It was also anticipated that the estimated effects of the PhACs would vary according to the microalgae growth parameters and indicators employed.

2 | MATERIALS AND METHODS

2.1 | Microalgae cultivation

The microalgae *R. subcapitata* (Korshikov) (Nygaard, Komárek, Kristiansen, & Skulberg, 1987; culture inoculum provided by the Ecotoxicology and Applied Ecology Group, NEEA-USP, São Carlos, São Paulo, Brazil) was cultured in a 250-ml Erlenmeyer flask with 100 ml of LC Oligo medium (Associação Brasileira de Normas Técnicas, 2018). The culture was kept at 25°C under a cool-white fluorescent lamp (intensity of 4,500 lux), with a 24-hr light to 0-hr dark photoperiod (Associação Brasileira de Normas Técnicas, 2018). The culture medium was previously sterilized for 15 min at 121°C, 1.05 kg/cm^2. Previous tests were carried out to check the growth phases of the microalgae (Figure S1). After 20 days of acclimatization, 20-ml aliquots of the culture were distributed to several sterile 50-ml glass flasks for the toxicity assays, which were performed in triplicates. The cell density was determined using an inverted microscope at ×400 magnification (Model BX51; Olympus, Japan) and Fuchs-Rosenthal counting chambers. The cell counts were correlated with the optical density (OD) at 680 nm (Ma et al., 2006), measured with a spectrophotometer (Model DR/4000V; Hach). Satisfactory linear relationships were obtained between the cell densities and the OD values (R^2 = .999, data not shown).

Toxicity tests using sodium chloride (NaCl) as a reference substance were performed to evaluate the physiological conditions of *R. subcapitata*, considering a 96-hr exposure period (Mansano et al., 2017).

2.2 | Protocol for exposure of *R. subcapitata* to the PhACs alone and in combination with caffeine

Standards of ABZ (>98%) and CAF (ReagentPlus, 98%) used in the experiments were purchased from Sigma-Aldrich. CBZ (>98%) was purchased from Toronto Research Chemicals (Canada).

Stock solutions at 1,000 mg/L were prepared by dissolving ABZ in dimethylformamide, CBZ in methanol, and CAF in deionized water. These solutions were diluted with water to the desired concentrations for the assays. The final concentrations of dimethylformamide and methanol were 0.05% (v/v) and 0.5% (v/v), respectively, which are considered nontoxic to microalgae, as shown by Cho, Jeon, Pham, Vijayaraghavan, and Yun (2008) for the Chlorophyta *Selenastrum capricornutum*, considering a concentration of 1.2% (v/v) for both solvents and inhibition of the growth rate as the evaluation criterion. The microalgae were exposed to nominal concentrations of 1, 5, 10, 50, 100, and 500 µg/L of ABZ and CBZ individually. For the mixture assays, the same range of nominal concentrations for ABZ and CBZ was used, but with the addition of 50 µg/L of CAF (also nominal concentration) in each assay (ABZ-C and CBZ-C, respectively). Stability tests for ABZ, CBZ (500 µg/L), and CAF (50 µg/L), as well as the formation of ricobendazole, were conducted under the experimental conditions of the assays in the LC Oligo medium. The concentrations of each PhAC in such tests were determined using a validated online solid-phase extraction ultrahigh performance liquid chromatography–tandem mass spectrometry method (SPE–UHPC–MS/MS). Details of the method are provided in the Supporting Information Material (Table S1 and Figure S2). The intraday precision was below one-half of the acceptable values given by the Horwitz function (27.9% for 25 µg/L and 22.6% for 100 µg/L) (González, Herrador, & Asuero, 2010). The stability remained between 91.3 ± 5.9% and 107.9 ± 0.0% for ABZ, 92.9 ± 1.8% and 110.5 ± 1.1% for CBZ, and 98.4 ± 7.3% and 115.6 ± 1.6% for CAF during the 16 days.

Control groups (blanks) were included, using the LC Oligo medium and the microalgae culture, but without any PhAC. The growth rates (Equation 1) of the microalgae were indirectly estimated daily for 16 days, using the OD measured at 680 nm and its linear relationship with cell density. Maximum cell density (cells/ml) (A), growth velocity (day^{-1}) (μ_{max}), and lag time (day) (λ) were calculated using the modified Gompertz function (Equation 2; Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990). All the modified

Gompertz function fits resulted in determination coefficients (R^2) higher than .974.

$$\text{Growth rate} = \frac{\sum_{i=0}^{16} \frac{\ln(Y_i)}{\ln(Y_0)}}{t_a}, \quad (1)$$

where Y_i is the cell density (cells/ml) at Day i; Y_0 is the cell density (cells/ml) at Day 0; t_a is the total exposure time (16 days in this case).

$$\frac{\ln(Y_i)}{\ln(Y_0)} = A\exp^{-\exp^{\frac{\mu_{maxe}}{A}(\lambda - t)+1}}, \quad (2)$$

where A is the maximum cell density (cells/ml); μ_{max} is the growth velocity (day^{-1}); λ is the lag time (day); t is the assay time (day, from 0 to 16 in this case).

The concentrations of chlorophyll-a (Chl-a) were determined on the first and the last days of each experiment. The samples were filtered using a GF-3 membrane (0.45-μm porosity; Macherey-Nagel®), following ethanol (10 ml) extraction. The ODs of the solutions were measured at 665 and 750 nm, before and after acidification with 0.4 N HCl (Nusch, 1980), and the Chl-a concentrations were calculated using the following equation:

$$\text{Chl-a} = [(665_b - 750_b) - (665_a - 750_a)]\, 29.6\frac{V_e}{V_f d}, \quad (3)$$

where 665_b and 750_b are the optical densities at 665 and 750 nm before acidification, respectively; 665_a and 750_a are the optical densities at 665 and 750 nm after acidification, respectively; V_e is the volume of ethanol (ml); V_f is the volume of the filtered culture sample (L); d is the thickness of the cuvette (1 cm).

2.3 | Statistical analyses

The growth velocity and lag time data were log-transformed (Organization for Economic Cooperation & Development [OECD], 2011), so that all the data followed normal distribution (Kolmogorov–Smirnov test). One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to determine whether the results of the exposure assays were significantly different ($p < .05$) in comparison to the control group and among the PhAC assays (with and without caffeine). The experimental data were processed using Origin 2019® software. Tables providing the detailed statistical results are available as Supporting Information Material. The EC_{50} values [corresponding to 50% of the observed effect when compared with the control group (R_{esp})] were calculated for chlorophyll-a production, growth rate, and maximum cell density by plotting the effect against the natural logarithm of the test substance concentration (Equation 4; W. Zhang et al., 2012). The $EC_{50-72\,hr}$ values for the growth rates were calculated according to the OECD 201 guideline (OECD, 2011). The complete data are available as Supporting Information Material (Tables S2 and S3).

$$R_{esp} = a + \ln(C)b, \quad (4)$$

where R_{esp} is the observed effect relative to the control; a and b are model parameters; and C is the concentration of the tested substance (μg/L).

2.4 | Mixture model

The independent action model (Equation 5, Cleuvers, 2005) was used to predict the effect of the combination between ABZ or CBZ with CAF. For this reason, previous test with CAF alone was conducted under the same conditions described above, and the effects (R_{esp}, same of Equation 4) of 50 μg/L of CAF to Chl-a, growth velocity, growth rate, and maximum cell density were estimated as 60%, 34%, 37%, and 45%, respectively.

$$E_{mix} = 1 - [(1 - E_{c1})(1 - E_{c2})], \quad (5)$$

where E_{mix} is the predicted effect of the mixture (ABZ + CAF or CBZ + CAF); E_{c1} and E_{c2} are the observed effects of PhACs alone (ABZ and CAF alone, respectively, or CBZ and CAF alone, respectively).

The comparison between the predicted effect and the observed effect was carried out according to the effect residual ratio (ERR; Equation 6; Wang, Liu, Zhang, & Li, 2010). As ERR data had normal distribution (Kolmogorov–Smirnov test), t- test was used to evaluate the deviation between predicted and observed effects and thus the characteristics of the interactions (i.e., positive values indicated a trend toward antagonistic interactions, and negative values suggested synergistic interactions; Gao, Feng, Kang, Xu, & Zhu, 2018).

$$\text{ERR} = \frac{E_{mix} - E_{obs}}{E_{mix}}, \quad (6)$$

where E_{obs} is the observed effect of the combination of PhACs (ABZ + CAF or CBZ + CAF).

3 | RESULTS

3.1 | Stability of the pharmaceuticals under the assay conditions

All the PhACs remained stable during the 16 days, reflecting their inherent recalcitrance. In addition, there was no relevant oxidation of ABZ to ricobendazole (Table S4). However, it should be noted that in the presence of the microalgae, the concentrations of the PhACs could decrease during the tests, representing a limitation of the present study.

3.2 | Effects on *R. subcapitata* of ABZ, carbamazepine, and their combinations with caffeine

The reference tests using NaCl indicated that the sensitivity of *R. subcapitata* ($EC_{50-96\,hr}$ = 70 mmol/L) was within the expected

range (reference range: 29.8–76.9 mmol/L) after 96 hr of exposure (Mansano et al., 2017).

The *R. subcapitata* growth velocity was significantly inhibited ($p < .05$; ANOVA with Tukey's post-hoc test) by ABZ at all the concentrations tested 1–500 µg/L), in comparison to the control group, while CBZ only had a significant effect at concentrations equal to or above 10 µg/L (Figures 1a and S3A). For the concentrations tested, the lag times did not exhibit any clear pattern of statistical similarity or difference, in comparison to the control group. At low concentrations (1, 5, and 10 µg/L), ABZ seemed to accelerate the exponential growth phase of the microalgae, compared with the control ($p < .05$). In most cases, CBZ did not affect the lag time (Figure S3B).

Overall, the Chl-a concentrations, growth rates, and maximum cell densities were significantly reduced ($p < .05$) in all assays, compared with the control group (Figures 1b–d and S3C–E). The EC_{50} values showed that in the individual assays, ABZ was more toxic than CBZ to *R. subcapitata*, affecting the growth rate and the maximum cell density. For these parameters, the EC_{50} values for ABZ were 5.8 and 4.8 lower than for CBZ, respectively. In contrast, for Chl-a, the EC_{50} value was 1.9 times higher for ABZ than for CBZ (Table 2).

The addition of CAF significantly lowered ($p < .05$) the effects of ABZ or CBZ on Chl-a production by *R. subcapitata*, for all the concentrations tested (Figures 1b and S3C). This was confirmed by the increase of EC_{50} of 3.1 and 12.7 times for ABZ and CBZ, respectively, when CAF was present (Table 2). CAF also increased the maximum cell densities at low ABZ concentrations (1–50 µg/L) and at high CBZ concentrations (50–500 µg/L) (Figures 1c and S3D). Again, EC_{50} increases of 3.7 and 2.0 times for ABZ and CBZ, respectively, were observed in the presence of CAF (Table 2). In general, CAF did not significantly influence the growth velocity and lag time of *R. subcapitata* in the ABZ and CBZ assays (Figures 1a and S3A,B). The microalgae growth rates were significantly increased by CAF for 1 and 50 µg/L of ABZ and for all concentrations of CBZ ($p < .05$) (Figures 1d and S3E). In addition, EC_{50} increases of 1.8 and 4.0 times were observed for ABZ and CBZ, respectively (Table 2). In summary, CAF reduced the negative effects of ABZ and CBZ for the parameters Chl-a, growth rate, and maximum cell density of *R. subcapitata*.

A comparison of the EC_{50} values for the growth rates, considering different exposure times (Tables 2 and S3) suggested that for the longer exposure time, the EC_{50} values were lower for ABZ (90.8 nmol/L for 72 hr and 29.4 nmol/L for 16 days) and higher for CBZ (105.5 nmol/L for 72 hr and 170.3 nmol/L for 16 days).

The independent action models suggested that the combinations ABZ + CAF and CBZ + CAF had antagonistic interactions (Figure 2) in comparison to the effects of the individual compounds. The ERR values were significantly positive ($p < .05$; *t*- test) for all tested parameters [e.g., growth velocity (Figure 2a), Chl-a (Figure 2b), maximum cell density (Figure 2c), and growth rate (Figure 2d)]. The full modeling results are also presented in the Supporting Information Material (Table S7).

4 | DISCUSSION

R. subcapitata is a representative of the Sphaeropleales group and has the potential to adapt to various environmental conditions, so it is considered an important test species for assessment of the effects of contaminants in the aquatic environment (Suzuki, Yamaguchi, Nobuyoshu, & Kawachi, 2018). Due to the adaptiveness of this microalgae and its sensitiveness for many compounds, *R. subcapitata* is widely used in ecotoxicological assays (Carusso, Juárez, Moretton, & Magdaleno, 2018). However, it is possible that an adaptation of the microalgae to the experimental conditions, especially in long-term exposure assays, can lead to underestimation of the observed effects. The present study contributes to understanding of the long-term effects of three widely used PhACs toward the microalgae, which can serve as a starting point for the delineation of water contamination scenarios and the promotion of risk assessment initiatives.

4.1 | Effects of ABZ and carbamazepine on various aquatic organisms

The effects of ABZ in cells are relatively well known. It acts as an inhibitor of succinate dehydrogenase (SDH) and glucose transport, which are associated with adenosine triphosphate synthesis (Gao et al., 2007). ABZ may also inhibit glutathione *S*-transferase (GST; a Phase II metabolism enzyme involved in the biotransformation of PhACs) and acts as a catalyst of PhACs conjugation, assisting excretion of the compounds and mitigating the effects of reactive oxygen species (ROS; Aguirre-Martínez, Delvalls, & Martín-Díaz, 2015; Gao et al., 2007). Carlsson, Patring, Kreuger, Norrgren, and Oskarsson (2013) observed that ABZ (at 173.4 nmol/L) caused 50% mortality of zebrafish (*Danio rerio*) embryos. Oh et al. (2006) reported an EC_{50} (48 hr) of 255.9 nmol/L for ABZ, using *Daphnia magna*. Fagundes (2018) observed that ABZ at 3.3 µmol/L caused 30% growth rate inhibition of *R. subcapitata* in 96-hr ecotoxicological assays. Escher et al. (2008) reported that 113.1 µmol/L of ABZ did not inhibit photosystem II in the microalgae *Desmodesmus subspicatus* during 24-hr ecotoxicological assays. The present study is one of the first to assess the effects of ABZ on the growth and production of chlorophyll-a in microalgae. Although other studies have reported the effects of antimicrobials on the growth rate of *R. subcapitata*, the exposure times were generally shorter (e.g., 72 and 96 hr; Table 3) than employed here (16 days).

SDH catalyzes the oxidation of succinate to fumarate in the mitochondria matrix and allows a rapid transfer of electrons to ubiquinone. Also, the loss of SDH in plant cells can induce the production of

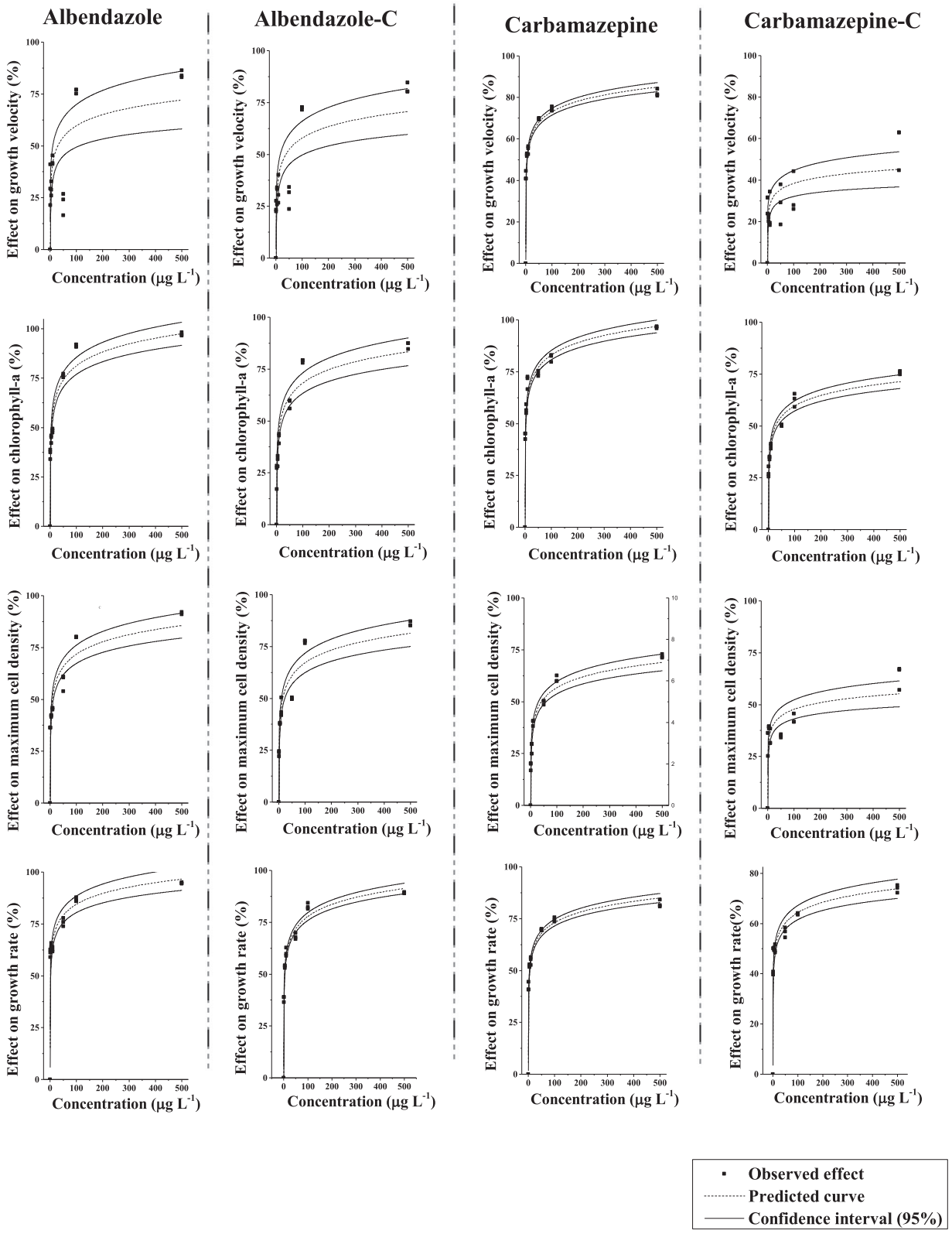


FIGURE 1 Dose–response curves (with 95% of confidence intervals) for growth velocity, chlorophyll-a, maximum cell density, and growth rate for *Raphidocelis subcapitata* after 16 days of exposure to albendazole (ABZ) alone (first column), ABZ with caffeine (second column), carbamazepine alone (third column), and carbamazepine with caffeine (fourth column). Full modeling parameters are presented in the Supporting Information Material (Table S6)

TABLE 2 Effective concentrations (EC_{50}; 95% confidence interval) and corresponding R^2 values for the toxicity tests with three different PhACs, considering the parameters: chlorophyll-a production, growth rate, and maximum cell density of *Raphidocelis subcapitata*

	Chlorophyll-a production (μg/L)		Growth rate (day^{-1})		Maximum cell density (cells/ml)	
PhAC	EC_{50} (nmol/L)	R^2	EC_{50} (nmol/L)	R^2	EC_{50} (nmol/L)	R^2
Albendazole	16.6 (11.7–22.6)	.933	29.4 (20.7–43.3)	.917	3.7 (1.9–5.6)	.901
Albendazole-C	52.0 (33.9–79.2)	.902	53.2 (35.8–81.0)	.901	13.6 (11.3–15.1)	.981
Carbamazepine	8.7 (8.5–10.6)	.974	170.3 (122.8–244.0)	.943	17.8 (14.8–21.2)	.981
Carbamazepine-C	110.1 (84.7–146.1)	.958	677.7 (na)	.721	36.0 (25.4–52.9)	.918

Note: The suffix "-C" indicates the presence of caffeine.
Abbreviations: PhAC, pharmaceutically active compound; na, not available.

ROS in the mitochondria, damaging the organelle (Huang & Millar, 2013). GST, in turn, plays an important role in the detoxification system, accelerating the polarization of the compound and, consequently, the elimination of this toxic compound from the cell (Kirchhoff & Pflugmacher, 2000). In the present study, the inhibition of SDH and GST (and the possible induction of ROS production and inhibition of the polarization of the PhACs), could explain the higher toxicity of ABZ than CBZ to *R. subcapitata*, affecting the growth rate and the maximum cell density. At levels of 211.8 nmol/L or higher, CBZ actually stimulates GST (W. Zhang et al., 2012). Using 24-hr assays, Vernouillet et al. (2010) reported that 6.8% of CBZ in the environment could be accumulated and transferred to the food chain by *R. subcapitata*. Aguirre-Martínez, Owuor, et al. (2015), Ferrari, Paxéus, Giudice, Pollio, and Garric (2003), and Di Poi, Costil, Bouchart, and Halm-Lemeille (2018) observed effects of CBZ at concentrations in the range from 2.5 to 682.7 μmol/L on the growth rate of *R. subcapitata* during short-term exposures (such as 72 and 96 hr). CBZ induces a rapid response of catalase and superoxide dismutase enzymes, both enzymes are part of the first line of defense against the ROS toxicity in the cell (W. Zhang et al., 2012). We speculate that these inductions may partially explain the lower toxicity of CBZ to growth rate and maximum cell density of *R. subcapitata* compared with ABZ.

CBZ can inhibit the synthesis of protochlorophyll and its subsequent conversion to chlorophyll-a, as already described for *Scenedesmus obliquus* and *Chlorella pyrenoidosa* (W. Zhang et al., 2012). As ABZ does not have a specific mechanism of action with regard to Chl-a, the inhibition of protochlorophyll could provide an explanation for the greater deleterious effect of CBZ on Chl-a as observed here. Furthermore, CBZ induces lipid peroxidation, which is a critical consequence of ROS production (Aguirre-Martínez, Delvalls, et al., 2015). This mechanism of oxidative stress in the cells may explain the toxicity of CBZ to *R. subcapitata* and its effects on the growth parameters and Chl-a observed here.

4.2 | Importance of the exposure time in ecotoxicological evaluations

The United Nations Economic Commission for Europe (UNECE) defines acute and chronic aquatic toxicity according to the exposure time related to the life cycle of the organism. Both acute and chronic aquatic toxicities are an intrinsic property of a substance that causes effects in a specific organism. However, short-term exposure is considered for acute assessments, while long-term exposure is used for chronic assessments (UNECE, 2017). Regarding ecotoxicological evaluations with microalgae—organisms with shorter generation times in comparison to other aquatic organisms—UNECE (2017) defines that assays with a duration of 72–96 hr are representative of acute assessments. Thus, in the present study, assays with exposure times longer than 96 hr were considered chronic assessments.

Most studies reported in the literature employed relatively short exposure times (such as 24, 72, or 96 hr; Table 3). However, due to the chronic inputs of PhACs to many water bodies, microalgae are continuously exposed to these compounds, making it necessary to evaluate the effects of long-term exposure. Jos et al. (2003) reported acute toxicity of CBZ (48-hr exposure; EC_{50} of 19.1–1,624.3 μmol/L) in aquatic organisms (*Chlorella vulgaris*, *D. magna*, and *Vibrio fischeri*) at micromoles per liter levels, which are above than usually expected environmental concentrations. Moreover, W. Zhang et al. (2012) reported that CBZ only inhibited the growth rates of *S. obliquus* and *C. pyrenoidosa* after 48 hr of exposure, considering concentrations at the micromoles per liter level (EC_{50} of 309.0 and 1,016.1 μmol/L, respectively). However, the authors observed that with longer exposure times (from 2 to 30 days), the EC_{50} decreased to 3.4 μmol/L, more consistent with the present results after 16 days of exposure (Table 3). In another study, it was reported that CBZ at 42.3 nmol/L caused 53.5% inhibition of Chl-a in *Navicula* sp. after 72 hr of exposure (Ding, Lin, Yang, & Li, 2019). In the present study, the results were of the same order of magnitude (EC_{50}: 8.7 nmol/L), despite the use of a different microalga and a longer exposure time. In general, longer exposure times lead to lower EC_{50} values, as observed here for *R. subcapitata* and by W. Zhang et al. (2012) for *S. obliquus* and *C. pyrenoidosa*.

4.3 | Caffeine reduces the toxicity of ABZ and carbamazepine to *R. subcapitata*

Ecotoxicological assays exploring the effects of exposure to combined PhACs are still scarce in the literature. In experiments with

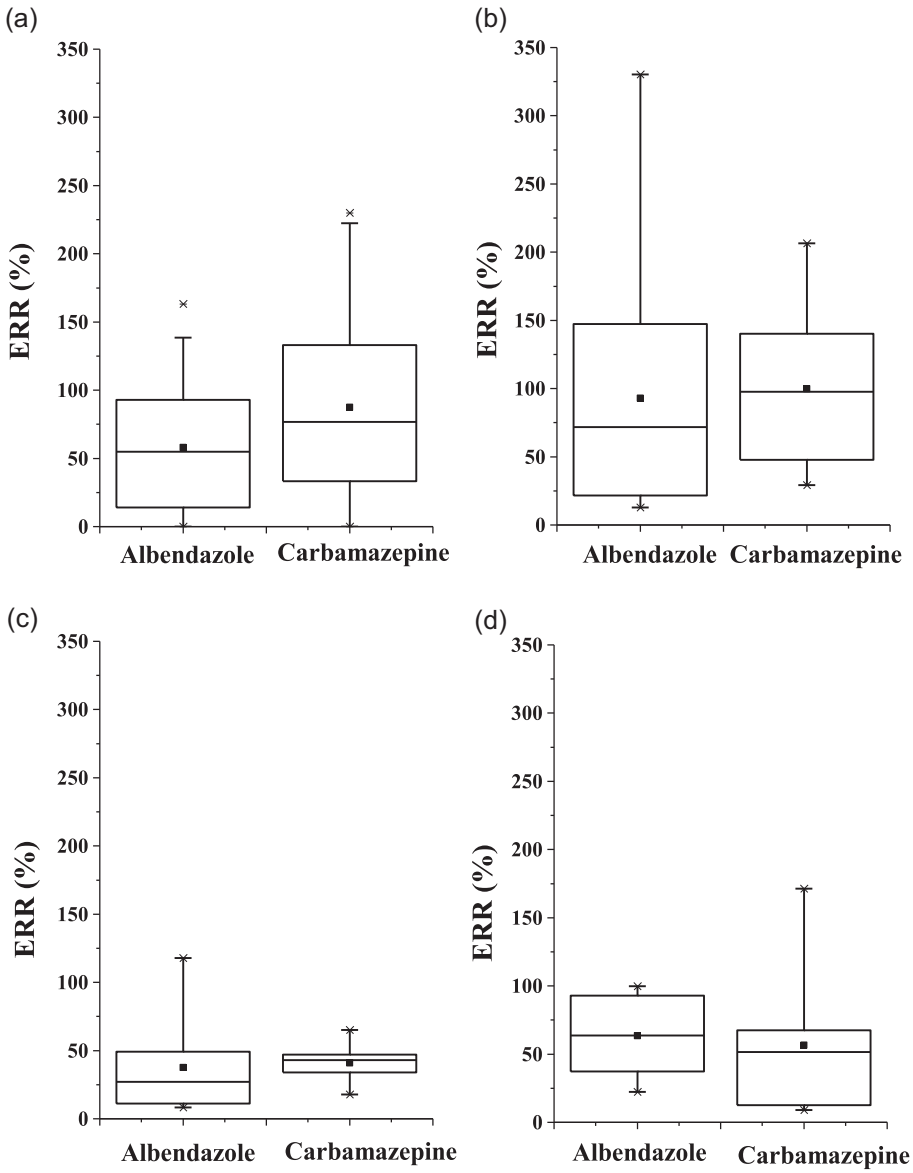


FIGURE 2 Effect residual ratio (ERR, %) for (a) growth velocity, (b) chlorophyll-a, (c) maximum cell density, and (d) growth rate of *Raphidocelis subcapitata* after 16 days of exposure to the combination between ABZ or CBZ with CAF. Average values are given by the black squares, whereas bottom, center, and top of boxplots show the 25th, 50th, and 75th percentiles, respectively. Bottom and top whiskers represent the 5th and 95th percentiles, respectively

river mesocosms in Canada, Lawrence et al. (2012) observed that the combination of acetaminophen and CAF significantly reduced cyanobacterial biomass in the biofilms. Cao, Goldhan, Martin, and Köhler (2013) suggested that CAF could lower the toxic effects of sublethal concentrations of ampicillin and captopril on the growth rate of *Escherichia coli*. The results were analogous to those found here, as CAF reduced the toxicity caused by ABZ and CBZ in terms of the growth parameters of *R. subcapitata*.

The effects of CAF have been described for various biological communities. Aguirre-Martínez, Delvalls, et al. (2015) found that the presence of CAF induced the production of detoxification metabolism enzymes against PhACs, as well as the production of GST, in the clam *Corbicula fluminea*. According to the same authors, synthesis of the glutathione peroxidase and reductase enzymes that are components of cellular antioxidant systems was favored in the presence of CAF at concentrations of around 15–50 μg/L. Cao et al. (2013) highlighted that sublethal doses of CAF could stimulate metabolic activity, leading to increased cell growth of *E. coli*. These observations could help to explain the effects of CAF observed here, with decreases of the toxic effects of ABZ and CBZ to the microalgae *R. subcapitata*. Also, the independent action model applied in this study highlighted that CAF had antagonistic interactions with ABZ and CBZ relative to the effects of the individual compounds.

TABLE 3 Reported effects of individual PhACs on the growth rates of different microalgae

PhAC	EC_{50} (nmol/L)	Time of exposure (hr)	Microalgae	Category	References
Triclosan	1.8	72	*Raphidocelis subcapitata*	Antimicrobial	Yang et al. (2008)
Triclocarban	53.9	72		Antibacterial	
Tylosin	229.2	72		Antibiotic	
Carbamazepine	850,571.8	24	*Scenedesmus obliquus*	Antiepileptic	W. Zhang et al. (2012)
	309,064.0	48			
	296,908.1	96			
	231,257.9	144			
	29,182.5	480			
	3,388.4	720			
	5,673,147.0	24	*Chlorella pyrenoidosa*		
	1,015,840.7	48			
	209,233.4	96			
	140,237.2	144			
	38,331.2	480			
	29,648.4	720			
	>423,549.3	72	*R. subcapitata*		Di Poi et al. (2018)
	>2,117,746.7	72			Aguirre-Martínez, Owuor, et al. (2015)
5-Fluorouracil	845.5	96	*R. subcapitata*	Chemotherapeutic	Zounková et al. (2007)
Oxytetracycline	3,258.0	96		Antimicrobial	Zounková, Klimesová, Nepejchalová, Hilscerová, and Bláha (2011)
Flumequine	1,722.8	96			

Abbreviation: PhAC, pharmaceutically active compound.

5 | CONCLUSIONS

In summary, ABZ and CBZ affected the microalgae under the experimental conditions employed, as reflected in the growth parameters (maximum cell density and growth rate) and Chl-a production. Each PhAC influenced the microalgae *R. subcapitata* in a different way: ABZ affected the growth parameters, while CBZ influenced the concentration of Chl-a. The combination with CAF lowered the toxicity of both ABZ and CBZ to *R. subcapitata*, in agreement with the expected antioxidant effects of CAF. Thus, as predicted by the independent action model, CAF had an antagonistic effect when combined to ABZ or CBZ, suggesting the beneficial effects of CAF in the combination exposures. Studies employing mixtures of PhACs and extended exposure times are essential in attempts to create exposure conditions that are more representative of environmental conditions, to evaluate the potential toxic effects of emerging contaminants in aquatic ecosystems. The use of microcosms and mesocosms is also recommended for reproducing environmental conditions, as well the use of other microalgae. Finally, the present observations highlight an important and complex issue that should be addressed in future ecotoxicological evaluations of PhACs, namely the different effects caused by exposure either to an individual compound or to a combination of different compounds. In the present work, it was found that such combinations could lead to antagonistic interactions between the PhACs, which reduced the effects of ABZ and CBZ on the growth and development of *R. subcapitata*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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