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Long-term ecotoxicological effects of ciprofloxacin in combination with caffeine on the microalga *Raphidocelis subcapitata*

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ABSTRACT

Ciprofloxacin (CIP) is an antimicrobial "pseudo-persistent" in aquatic ecosystems. Once dispersed in the water compartments, it can also affect the microalgae. Thus, the evaluation of its long-term ecotoxicological effects is necessary. CIP interactions with other pharmaceuticals are not well known. In this study, we investigated the toxic effects of CIP alone and combined with caffeine (CAF), using the modified Gompertz model parameters and the chlorophyll-a production of the microalga *Raphidocelis subcapitata* as endpoints, throughout a 16-day exposure assay. The exposure to CIP alone led to significant reductions of the growth rate and the cell density of the microalgae compared to control groups. The combination with CAF lowered the adverse effects of CIP to *R. subcapitata*. However, as the toxicity is dynamic, our results indicated that the toxic effects in respect to the studied endpoints changed throughout the exposure period, reinforcing the need for longer-term ecotoxicity assessments.

1. Introduction

Ciprofloxacin (CIP), a broad-spectrum antimicrobial of the fluoroquinolone family, is used worldwide in human and veterinary medicine. After administration, CIP is generally not completely metabolized in mammals, so this drug and/or its metabolites can reach and contaminate water bodies and other environmental compartments [1,2]. In addition, it is acknowledged that traditional processes used in wastewater treatment plants, such as activated sludge or upflow anaerobic sludge blanket reactors, present limited capacity for the removal of many pharmaceuticals [3], which can lead to the release of active compounds into aquatic ecosystems and their presence at ng L^{-1} and μg L^{-1} levels [4–6]. CIP is a recalcitrant compound with high stability in the environment, and up to 34 μg L^{-1} of CIP has been reported in hospital wastewater by Rodrigues-Silva et al. [4]. In addition, concentrations of CIP equal to or higher than 310 μg L^{-1} were found to inhibit the growth of bacteria in activated sludge aeration tanks [7].

The presence of antimicrobials in water bodies can enable the development of resistance genes in bacteria [8]. In addition, residues of antimicrobials in the aquatic environment can affect the aquatic biota other than bacteria, such as copepods, fish, macrophytes, and microalgae [9–15]. Thus, CIP has been associated with the shifts in the antioxidant enzymes from the exposed organisms [16,17], which can promote genotoxicity to bacteria (*Salmonella thyphimurium*) [18] and chronic toxicity to the microalgae *Chlamydomonas mexicana, Chlorella vulgaris*, *Chlamydomonas reinhardtii,* and *Raphidocelis subcapitata* [17,19]. Venancio et al. [9] reported a compilation of previously obtained data about the chronic toxicity of CIP for *R. subcapitata*, the EC_{50} (half maximal effective concentration) compiled by the author vary from up to 5,000.0 to more than 10,000.0 μg L^{-1} after 96 h of exposure. However, none of the presented studies were carried out under exposure times longer than 96 h. Therefore, the available data seem to be insufficient to cover all the possible impacts of this antimicrobial to the environment [13] due to the absence of ecotoxicity assays with longer exposure times that could cover different growth phases of this microalgae.

While toxic effects are usually determined for a specific trophic level of organisms, such investigations are still scarce for complex mixtures of

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different compounds [20–23]. Toxicity assessments of drug combinations [14,24] and long-term exposure assays [15] can be performed to achieve more realistic conditions and provide reliable information about the potential antagonistic and synergistic effects of pharmaceuticals [22,25]. However, there have been few reported toxicity studies concerning the temporal evolution of the EC_{50}, although it is expected that an increase of the exposure time should decrease the EC_{50} value [15,26,27]. However, Xiong et al. [19] observed the opposite (increase of the exposure time leading to increased EC_{50}) in experiments with CIP and microalgae. Therefore, more studies are required to fully elucidate the variation of EC_{50}, considering longer exposure periods and not only a single value representative of the average conditions experienced by the test organisms in the ecotoxicological assays.

Caffeine (CAF), a well-known methylxanthine, is the most consumed psychotropic agent in the world [28] and is usually reported at μg L^{-1} levels in surface waters. Peteffi et al. [29] reported the occurrence of CAF in Brazilian water bodies at up to 28 μg L^{-1}, and its consumption has been growing in recent years [30]. Al-Qaim et al. [31] reported CAF concentrations from 31.7 to 50.1 μg L^{-1} in surface waters in Malaysia. Komori et al. [32] reported that the predicted no-effect concentration (considering the EC_{50} values for crustaceans and algae) for CAF is 5.2 μg L^{-1}, which indicates a risk to aquatic biota, given the previously reported CAF concentrations in water bodies. Moreover, CAF has dose-dependent effects, with either positive effects at lower doses or harmful ones at higher doses [33]. Among the possible positive outcomes, CAF can act against oxidative stress induced by several compounds [25,34]. In addition, the chemical stability of CAF (with aquatic half-life between three and ninety days) and its high consumption rate enable this molecule to be used as a reliable marker for anthropogenic contamination [29].

The toxicological studies reported in the literature have generally been performed using a single toxicological endpoint (such as growth rate) and have rarely evaluated the toxic effects of drug combinations under long-term exposures [15]. Ecotoxicological assays with microalgae are particularly interesting once different endpoints can be used, such as growth parameters and production of antioxidant enzymes [25,35]. As microalgae comprise the base of the food chains, their use as test organisms has been considered for several xenobiotics, and the results can offer important insights on the expected effects in the environment as a whole [36,37].

In the present study, the hypothesis that the antioxidant properties and mechanisms of action of CAF could affect the toxicity of CIP was tested. The ecotoxicological effects of CIP were assessed considering it in isolation (CIP) and in combination with CAF (CIP-CAF). Different endpoints (maximum cell density, growth velocity, lag time, and chlorophyll-a production) were considered for evaluation of the toxicity of CIP and CIP-CAF towards the microalga *R. subcapitata*. In order to observe the effects through all the growth cycle of the organism, the exposure period (16 days) was more extended than typically proposed in guidelines and reported in the literature. The EC_{50} in terms of growth rate was calculated daily during the 16 days of exposure to determine the effect of exposure time on this parameter.

2. Material and methods

2.1. Microalgae cultivation

Raphidocelis subcapitata culture strains provided by the Ecotoxicology and Applied Ecology Group (NEEA-USP, São Carlos, São Paulo, Brazil) were cultured in LC Oligo medium broth at 25 °C, under a fluorescent lamp (4,500 lux), without a dark photoperiod [38]. After an acclimatization period of 20 days, the culture was distributed into 50 mL glass flasks containing one part of culture to nine parts of LC Oligo medium with a total of 20 mL. The linear relationship of optical density (OD) at 680 nm and cell density was used to determine the microalgae growth. The culture broth and glass flasks were previously sterilized for 15 min at 121 °C and 1 atm. Microalgae such as *Raphidocelis subcapitata* have been widely used as test organisms, because they are primary producers and important players of nutrient cycling in aquatic ecosystems, so negative impacts against these organisms may bring relevant ecological implications [39].

R. subcapitata was exposed to sodium chloride (NaCl) at concentrations between 0.5 and 7.0 g L^{-1}, using a test solution dilution rate of 1.7, in accordance with the recommended dilution rate range from 1.2 to 2.0 [38]. This procedure was used to assess the physiological conditions of the organism and to validate the subsequent ecotoxicological tests. The exposure of the microalgae resulted in an $EC_{50-96\ h}$ value (the concentration causing 50 % inhibition of the growth rate after 96 h) of 4.10 g L^{-1} of NaCl (Fig. S1). The $EC_{50-96\ h}$ value obtained was in agreement with previous studies, such as the work by Mansano et al. [40], who reported $EC_{50-96\ h}$ values from 1.74 to 4.49 g L^{-1} of NaCl.

2.2. Protocol of toxicity assays with R. subcapitata exposed to ciprofloxacin and caffeine

Standards of CAF (Reagent Plus, 98 %) and CIP (>98 %) were purchased from Sigma-Aldrich (USA). Stock solutions (1,000 mg L^{-1}) were prepared by dissolving CIP in methanol (with 0.2 % v/v of acetic acid) and CAF in ultrapure water. The working solutions were prepared by continuous dilution in water until reaching the desired concentrations for the assays. The final methanol concentrations were <0.5 % (v/v) in all the aqueous test solutions. The exposure time and the initial cell density were selected based on Diniz et al. [25], ensuring that all growth stages of the microalgae have been covered. Preliminary control tests with methanol at 0.5 % (v/v) in water, under the same conditions intended for the ecotoxicological assessments, showed no adverse effects on the *R. subcapitata* growth parameters. The full data can be found in the Supplementary Material.

R. subcapitata were exposed separately to six nominal concentrations of CIP (1, 5, 10, 50, 100, and 500 μg L^{-1}) and CAF (15, 25, 50, 100, 500, and 1,000 μg L^{-1}), which were performed three times independently and each independent assay were performed with triplicates. The ranges for CIP and CAF concentrations were selected based on literature data for the environmental occurrence of these pharmaceuticals [4,29]. For the CIP-CAF combined assays, the same six nominal concentrations of CIP were added to 50 μg L^{-1} (nominal concentration) of CAF in each assay. The control group assays were performed only with LC Oligo medium and the microalgae. Throughout the test period (16 days), daily microalgae growth rates (Eq. (1); [41]) were indirectly obtained, as previously described by Diniz et al. [25]. The modified Gompertz model (Eq. (2)) was used to estimate the maximum cell density (cells mL^{-1}), growth velocity (day^{-1}), and lag time (day) [42]. All the modified Gompertz fits resulted in coefficients of determination (R^2) higher than 0.976.

$$\mu_{i-j} = \frac{ln\ X_j - ln\ X_i}{t_j - t_i}. \quad (1)$$

where, μ_{i-j} is the average specific growth rate from time i to time j (day^{-1}); X_i is the cell density at time i; and Xj is the cell density at time j.

$$\frac{ln(X_j)}{ln(X_0)} = A\ exp^{-exp^{\frac{\mu_e}{A}(\lambda - t)+1}} \quad (2)$$

where, A is the maximum cell density (cells mL^{-1}); μ_{max} is the growth velocity (day^{-1}); λ is the lag time (day); and *t* is the assay time (day).

The chlorophyll-a concentrations (Chl-a) were determined on the first and the last day of each experiment using filtration in a GF-3 membrane (Macherey-Nagel®, 0.45 μm) and extraction with ethanol (10 mL) during 12 h at 4 °C under dark conditions (Eq. (3)). The OD values of the solutions were measured at 665 and 750 nm, before and after acidification with 0.4 mol L^{-1} HCl [43]. The Chl-a concentrations were normalized by the number of cells for each case. All assays had the

same initial biomass content (663.0 ± 80.1 μg L^{-1} of Chl-a or 4.3 10^{-7} ± 0.5 10^{-7} μg $cell^{-1}$).

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

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

2.3. Statistical analyses

Since data had a normal distribution (Kolmorogov-Smirnov test), one-way analysis of variance (ANOVA), with Tukey's post-hoc test, was used to identify significant differences ($p < 0.05$) between the exposure and control groups, and between those with or without the presence of CAF. The Origin 2019® software was used to process all the experimental data. The EC_{50} values were calculated every 24 h for the growth rate (i.e., a new growth rate and a new EC_{50} were calculated every 24 h) by plotting the response versus the natural logarithm of the test substance concentration [41]. Only the data with R^2 ≥0.600 were considered.

3. Results and discussion

Although microalgae are exposed to different pollutants during their different growth phases in aquatic ecosystems, most ecotoxicological evaluation guidelines and studies consider short exposure times (72–96 h) and only the exponential growth phase of the microalgae. The latter consideration implies that the microalgae have full development capacity, with constant growth and abundant nutrients in the medium [7,12,19,38,41,44,45]. In this study, *R. subcapitata* was exposed to CIP and CAF during all its growth phases, resulting in a reliable ecotoxicological assay that better reflected real environmental conditions. Furthermore, given the recalcitrance of CIP, the use of longer exposure times could provide a better understanding of the toxic effects of this class of pharmaceuticals in the aquatic environment. In addition, different endpoints

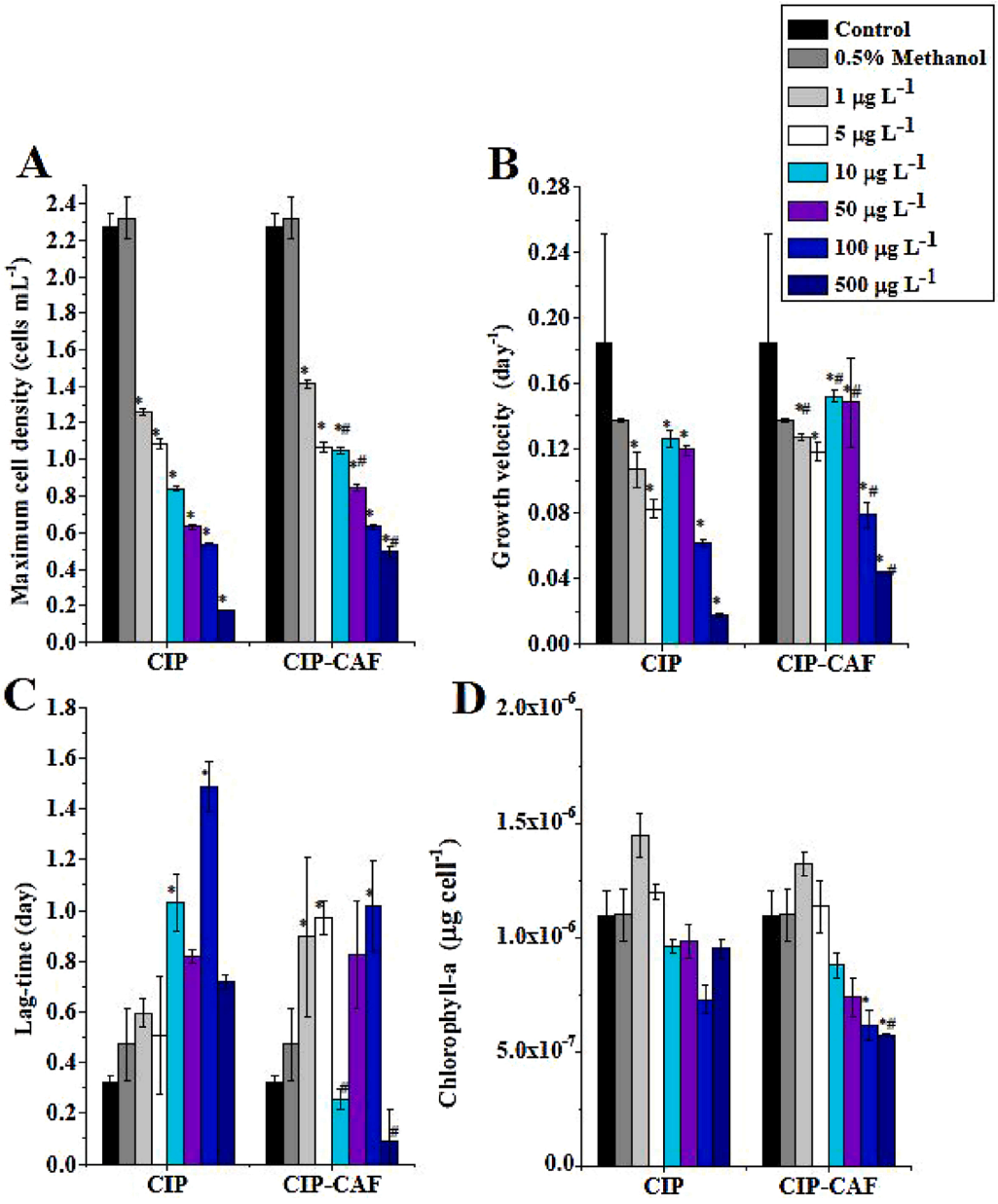

Fig. 1. Maximum cell density (A), growth velocity (B), lag time (C), and chlorophyll-a concentration (D) for *R. subcapitata* after 16 days of exposure to ciprofloxacin alone (CIP) and in combination with caffeine (CIP-CAF). Each column represents the mean of three independently assays, each one with its own triplicates; the error bars indicate the standard deviation; * and # indicate significant difference ($p < 0.05$) of the observed data for each treatment compared to the control group and compared to the presence of caffeine, respectively. The full data are available in the Supplementary Material (Table S2).

may assist in elucidating the mechanisms of toxicity of pharmaceuticals towards the organisms tested. It should also be highlighted that this study is the first to evaluate the potential uptake of CIP and its long-term effects on the development of the microalga *R. subcapitata*.

3.1. Effects of ciprofloxacin on R. subcapitata growth parameters

The maximum cell density and growth velocity of *R. subcapitata* were significantly inhibited ($p < 0.05$) by all the concentrations of CIP (from 1 to 500 μg L^{-1}), compared to the control group (Fig. 1A). The presence of CIP decreased the maximum cell density by up to 13-fold (at 500 μg L^{-1}). The growth velocity was affected even when the microalgae were exposed to the lowest CIP concentration, with a 1.7-fold decrease at 1 μg L^{-1} (Fig. 1B). The highest concentration of CIP (500 μg L^{-1}) led to a 10.2-fold decrease of the microalgae growth velocity. The lag time (Fig. 1C) showed no clear pattern of similarity or difference, compared to the control group. The use of CIP at 10 and 100 μg L^{-1} slowed the exponential growth phase (by 3.2 and 4.6-fold, respectively) of the microalgae, compared to the control ($p < 0.05$). The normalized Chl-a concentration was not affected by the CIP tested concentrations, compared to the control group (Fig. 1D). In general, CIP significantly ($p < 0.05$) inhibited the daily growth rate of the microalgae. The growth rate full dataset for *R. subcapitata* exposed to CIP during 16 days are presented in Table S1 (Supplementary Material).

A hypothesis to explain the toxicity based on the observed growth parameters (growth rate, growth velocity, and maximum cell density) of the microalgae could be attributed to a possible increase in the concentration of malondialdehyde (MDA), a product of the peroxidation of polyunsaturated fatty acids (PUFA), as already reported by Xiong et al. [19]. The authors observed that the exposure of the microalga *C. mexicana* to increasing concentrations of CIP led to higher concentrations of MDA in the medium, causing cell damage. Similar toxic effects were observed for *C. vulgaris* and *C. reinhardtii* [19]. Furthermore, in addition to its antimicrobial activity, CIP may act in the inhibition of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which are present in other microorganisms [46]. Aderemi et al. [17] assessed the effects of CIP to *R. subcapitata* in 120-h exposure assays. The authors observed that, as reported by Xiong et al. [19] to *C. Mexicana*, *C. vulgaris*, and *C. reinhardtii*, the presence of CIP induced a 1.4 higher content of MDA compared to the control groups. In addition, Aderemi et al. [17] reported that higher concentrations of CIP inhibited the production of SOD probably due to a high accumulation of the superoxide radical. The same pattern was observed by Nie et al. [47] in a study with *R. subcapitata* and CIP. Aderemi et al. [17] also reported that CIP reduced the available energy for *R. subcapitata* growth since this pharmaceutical induced energy overconsumption by the microalgae. Regarding Chl-a concentration, Aristilde et al. [48] reported that despite CIP could inhibit the key enzymes involved in the photosystem II electron transport, the lowest observed effect concentration (LOEC) was 3.3 mg L^{-1}, which is 6.6 times higher than the highest concentration used in the present study. These reported mechanisms validate the results obtained in the present study and may explain the toxic effects of CIP to the *R. subcapitata*.

Considering the assays with CIP (used alone), the range of EC_{50} values (Fig. 2) varied from 5.4 μg L^{-1} (day 2) to 48.4 μg L^{-1} of CIP (day 8). The lower EC_{50} values observed in the first days could also have been due to the lag phase, with the microalgae adapting to the medium, so a low or even negative growth rate would be expected [49]. Xiong et al. [19] reported a variation of the EC_{50} for *C. mexicana* exposed to CIP, with the values increasing over the course of 11 days, which was suggested to be due to CIP uptake (13.0 %) by the microalgae. The same study found that some other microalgae species (*Chlamydomonas pitschmann*, *C. vulgaris*, and *Ourococcus multiporus*) could assimilate antimicrobials (2.0 %) such as CIP from the medium. In our study, after day 8, a constant decrease of EC_{50} was observed, with a decrease from 48.4 μg L^{-1} (day 8) to 6.4 μg L^{-1} (day 16) (Fig. 2). It was reported that the

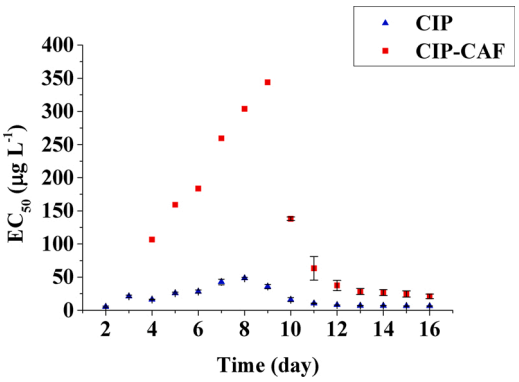

Fig. 2. Evolution of EC_{50} (for growth rate) during 16 days of *R. subcapitata* exposure to ciprofloxacin alone and in combination with caffeine. The error bars indicate the calculated standard deviations. Only R^2 equal to or above 0.600 was considered. The full data are available in the Supplementary Material (Table S3).

presence of CIP in the culture medium induces an overaccumulation of lipids and proteins, as well as an increase in the MDA content in *R. subcapitata* [17]. These observations are important since the microalgae accumulate lipids when exposed to CIP and, consequently, the cells become more susceptible to oxidation by MDA. Cell lysis in the presence of CIP (due to the increase of MDA) could lead to the release of previously assimilated CIP (and probably also CIP metabolites) into the medium, consequently affecting the development of the microalgae.

3.2. Effects of caffeine on R. subcapitata growth parameters

The *R. subcapitata* maximum cell density showed significant inhibition ($p < 0.05$) by exposure to all the concentrations of CAF (from 15 to 1,000 μg L^{-1}) (Table S2, Fig. S2). At 1,000 μg L^{-1}, CAF decreased the maximum cell density by 5.3 times. Meanwhile, at the same concentration, CAF significantly ($p < 0.05$) increased the normalized Chl-a content at each cell by 1.5 times (i.e., 11.0 10^{-7} μg $cell^{-1}$ to 16.2 10^{-7} μg $cell^{-1}$). However, the normalized Chl-a was not affected by CAF at the other tested-concentrations (15–500 μg L^{-1}). The growth velocity was only significantly affected ($p < 0.05$) at the highest concentrations of CAF (from 100 to 1,000 μg L^{-1}), while no effect was observed for the lag time. An influence of CAF on the *R. subcapitata* growth rate was also observed. The EC_{50} for growth inhibition after 16 days ($EC_{50-16\ days}$) was 154.9 μg L^{-1} (Table S3).

Zarrelli et al. [50] suggested that at mg L^{-1} levels, CAF does not affect microalgae specific growth rate, although this was based on experiments with microalgae in the exponential growth phase. In contrast, the present study exposed the microalgae during all the growth phases (lag, exponential, and stationary phases), which might explain the lower toxicity levels observed. Lawrence et al. [51,52] reported that microorganisms could use CAF and its byproducts as sources of carbon and nitrogen, with 10 μg L^{-1} of CAF promoting significant increases in the bacterial biomass of a riverine biofilm. In addition, CAF has antioxidant properties [53]. CAF has been found to induce the production of antioxidant species, such as cytochrome P450 enzymes (ethoxyresorufin-*O*-deethylase and dibenzylfluorescein) in Phase I of detoxification metabolism, as well as the enzymes glutathione S-transferase (GST), glutathione peroxidase (GPX), and glutathione reductase (GPR) in Phase II of detoxification metabolism [54]. Aguirre-Martínez et al. [54] reported that 15–50 μg L^{-1} of CAF induced GPX and GPR production in the clam *Corbicula fluminea*. Based on these effects, a CAF concentration of 50 μg L^{-1} was selected in the present study to evaluate its influence on the toxicity of ciprofloxacin towards *R. subcapitata*. In addition, 50 μg L^{-1} of CAF represented one-third of the $EC_{50-16\ days}$ determined in our study (154.9 μg L^{-1}, Table S4).

3.3. Effects of caffeine on the toxicity of ciprofloxacin towards R. subcapitata

The exposure of microorganisms to pharmaceuticals can cause excessive production of cytotoxic ROS, which may be lethal to cellular organelles due to their strong oxidation properties [19]. In our study, the effect of the antioxidant properties of CAF in reducing ROS were observed indirectly, since CAF lowered the toxicity of CIP towards *R. subcapitata*.

The growth parameters of *R. subcapitata* after 16 days of exposure to CIP-CAF are shown in the Supplementary Material (Table S5). The evolution of the EC_{50} values is summarized in Fig. 2. The EC_{50} was higher for the CIP-CAF assays, compared to the ones with CIP alone, which demonstrated that the addition of CAF to the medium inhibited the toxicity of CIP towards the microalgae. However, compared to the control assays, significant inhibition of the growth rate was observed at all concentrations of CIP ($p < 0.05$), even in the presence of CAF, from day 10 to day 16.

The CIP-CAF combination changed the microalgae growth inhibition dynamics during the first 9 days of the assay. The EC_{50} values increased from 106.7 μg L^{-1} (day 4) to 344.0 μg L^{-1} (day 9). The EC_{50} then decreased to 138 μg L^{-1} on day 10, reaching 21.1 μg L^{-1} on day 16 (Fig. 2). The increase of the EC_{50} in the first 9 days could have been due to CIP uptake by the microalgae. After day 9, the increase of MDA probably led to the release of intracellular CIP and its metabolites into the medium, hence explaining the decrease of the EC_{50}.

In the presence of CAF, the maximum cell density increased significantly ($p < 0.05$) in solutions containing CIP at concentrations above 10 μg L^{-1}, with a 2.8-fold increase for CIP at 500 μg L^{-1}. Significantly higher ($p < 0.05$) growth velocities were observed for the CIP-CAF assays, reaching a 2.5-fold increase for CIP at 500 μg L^{-1} (Fig. 1B). Overall, the presence of CAF did not induce faster development of the microalgae (Fig. 1 C), with no effect on the lag time, nor did it influence normalized Chl-a (Fig. 1D) compared to CIP alone.

To the best of our knowledge, the present study is one of the first to evaluate the effects of the combination of CIP and CAF. Vannini et al. [23], in a study with several mixtures of therapeutic drugs, including CIP, observed that drug combinations decreased the activity of adenosine triphosphate synthase beta-subunit in *R. subcapitata*, suggesting that drugs may interfere with energy transduction in the mitochondria and chloroplasts of microalgae. Magdaleno et al. [44] evaluated binary combinations of six antimicrobials, including CIP, in ecotoxicity assays using *R. subcapitata* as the test organism. During 72 h assays, synergistic effects were observed at low concentrations of binary combinations of CIP with cephalothin, and CIP with vancomycin, which could be explained by the inhibition of the enzyme gyrase by CIP, which affected DNA replication. Diniz et al. [25] observed that CAF with albendazole or carbamazepine resulted in antagonistic interactions in assays with *R. subcapitata*. In the present study, the decreased effect of CIP in the presence of CAF reinforced the importance of evaluating drug combinations, since different interactions (additive, synergistic, and antagonistic) could be observed.

Parsons and Surgeoner [26] were among the first to evaluate the effect of exposure time on EC_{50}. They observed that longer duration of exposure of *Aedes aegypti* mosquito larvae to insecticides led to lower EC_{50} values. The same pattern was observed by Ramos et al. [27] in studies with *Daphnia magna* (copepod), *Poecilia reticulata* (fish), and *Lymnaea stagnlis* (snail) exposed to polar narcotics. Zhang et al. [15] reported that longer exposure of different microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*) to carbamazepine reduced the EC_{50}. In contrast, in studies with CIP and the microalga *C. mexicana*, Xiong et al. [19] found that the EC_{50} increased with longer exposure times, due to the ability of the microalgae to recover from CIP toxicity. In the present study, the temporal increase and decrease of EC_{50} during the assays could probably be explained by CIP uptake and posterior cell lysis, since the 16-day experiments captured such longer-term dynamics involved in the interaction between the drugs and the microalgae. Also, as CIP reduces the energy available for the growth of *R. subcapitata* and induces higher energy expenditure per cell [17], it is plausible that longer exposure times result in lower growth rates as well as observed in our study.

The presence of CAF delayed the peak of EC_{50}, compared to the assays with CIP alone (Fig. 2). It is well known that CAF has antioxidant properties, inducing enzymes (GPR and GPX) that scavenge ROS, especially at CAF concentrations ≥50 μg L^{-1} [54]. The presence of CIP induces the production of MDA in *R. subcapitata* cells, which decreases membrane fluidity and increases the leakiness of the cells, due to ROS formation [17,47]. The induction of GPR and GPX decreases the effect of MDA and could provide an explanation for the delay observed in this study, due to the longer time required for cellular lysis. Further studies are needed to fully elucidate the variation of EC_{50} in ecotoxicological evaluations of CIP, CIP-CAF, and other pharmaceuticals, as well as the pattern of ROS overproduction, MDA formation, membrane permeability, and other antioxidant enzymes content. However, the present work is a first step towards more realistic ecotoxicological studies.

4. Conclusions

This work contributes to the understanding of the toxic effects of CIP towards the microalga *R. subcapitata*, as well as the antagonistic effects of CAF. Studies considering exposure times longer than the traditional 96 h recommended by the OECD [41] protocols allow the evaluation of the temporal evolution of ecotoxicity and provide more solid basis for risk assessment initiatives.

The modified Gompertz parameters and Chl-a production were suitable endpoints for the evaluation of CIP toxicity to the microalgae, under the protocol we employed. When CIP was tested alone, increase of the exposure time resulted in increase of the EC_{50} during the first eight days, followed by a subsequent decrease. This variation provides a new perspective in understanding the toxic effects of pharmaceuticals, showing that microalgae may react differently to pollutants, depending on their phase of growth. Shorter or longer exposure times can lead to different responses of microalgae exposed to pharmaceuticals, and processes as CIP uptake and cell lysis must be considered.

The presence of CAF lowered the toxicity of CIP to *R. subcapitata* and delayed the peak of EC_{50}, which could be attributed to the antioxidant properties of CAF. Ecotoxicological assays considering not only short-term exposures and isolated drugs are essential for obtaining representative assessments of the effects on aquatic biota. In the present work, we found that the toxic effects could vary according to the time of exposure, with the combination of the drugs altering the metabolism of the organisms, leading to different effects on the growth parameters and development of *R. subcapitata*.

CRediT authorship contribution statement

Vinicius Diniz: Conceptualization, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Gabriela Rath:** Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Susanne Rath:** Conceptualization, Validation, Supervision, Resources, Writing - original draft, Funding acquisition, Visualization. **Caio Rodrigues-Silva:** Validation, Investigation, Writing - original draft, Visualization. **José R. Guimarães:** Resources, Funding acquisition, Visualization. **Davi G.F. Cunha:** Conceptualization, Resources, Writing - original draft, Supervision, Project administration, Funding acquisition, Visualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.02.020.

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