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Effects of "COVID Kit" Drugs (Hydroxychloroquine, Azithromycin and Ivermectin) in the Freshwater Microalgae *Desmodesmus subspicatus*

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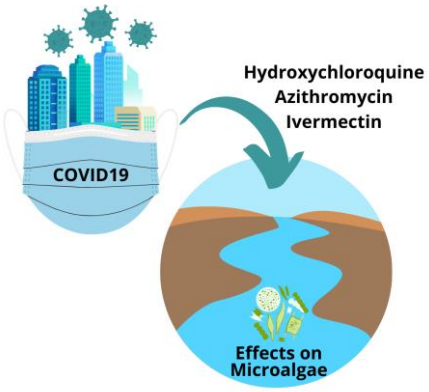
HIGHLIGHTS

- Microalgae were exposed to hydroxychloroquine (HCQ), ivermectin (IVM) and azithromycin (AZM).
- EC50 values were 510, >800 and 100 µg.L^{-1}, for HCQ, IVM and AZM, respectively.
- The highest AZM and mixture concentration inhibited microalgae growth.
- HCQ exposure increased CAT activity.

Abstract: The pandemic caused by the SARS-CoV-2 virus resulted in a significant increase in the consumption of drugs aimed at preventing and treating the disease, such as hydroxychloroquine (HCQ), ivermectin (IVM) and azithromycin (AZM). As a consequence, the presence of these pharmaceuticals increased in natural aquatic environments, posing risks to non-target organisms. In this study we aimed to determine the toxic effects of these drugs (72 h), at environmentally realistic concentrations (0, 1, 5, 10, 50 and 100 µg.L^{-1}), isolated or mixed, on the microalgae *Desmodesmus subspicatus*. The effective concentration capable to inhibit microalgae growth by 50% (EC50), final biomass production, chlorophyll a and b content, the activities of catalase (CAT) and glutathione S-transferase (GST), as well as the levels of malondialdehyde (MDA) and protein carbonyl (PC) were determined. EC50 values were 510, >800 and 100 µg.L^{-1}, for HCQ, IVM and AZM, respectively. The highest AZM and mixture concentration inhibited microalgae growth by 35.3 and 49.8%, respectively. HCQ caused an increase in CAT activity at concentrations of 5, 10 and 100 µg.L^{-1}, the other treatments did not cause significant changes. The data show that HCQ alters microalgae metabolism and the EC50 results indicate that HCQ and AZM are harmful to microalgae.

Keywords: hydroxychloroquine; ivermectin; azithromycin; EC50; biochemical biomarkers, *Desmodesmus subspicatus*.

GRAPHICAL ABSTRACT



INTRODUCTION

Contamination of natural aquatic environments by anthropogenic compounds is one of the problems arising from the human use of water resources, especially in densely populated areas [1]. As a consequence, substances such as pesticides, metals, hydrocarbons, hygiene and personal care products, and pharmaceutical compounds (PhaCs) are frequently found in the aquatic environment [2]. Products of pharmacological origin are widely consumed by humans, and most part of them are not completely removed by conventional sewage treatment systems. As a consequence, these compounds end up entering aquatic environments, causing negative effects to resident organisms [3].

The COVID-19 pandemic recently caused by the new coronavirus, resulted in an increase in sales of a group of specific PhaCs, known in Brazil as the “COVID Kit”, composed of hydroxychloroquine (HCQ), ivermectin (IVM) and azithromycin (AZM) [4, 106]. HCQ, belonging to the 4-aminoquinoline group and derived from chloroquine, is a drug commonly used as a treatment for malaria, rheumatoid arthritis and systemic lupus erythematosus [5]. IVM is an antiparasitic drug, belonging to the avermectin group, mainly used to treat diseases such as filariasis, strongyloidiasis, scabies and pediculosis [6]. AZM is a macrolide antibiotic used mainly to treat gram-positive bacteria such as *Streptococcus pneumoniae*, which causes pneumococcal pneumonia [7]. These three drugs were widely publicized as a form of prevention and/or treatment for COVID-19 is numerous countries, such as Brazil, even without scientific proof of effectiveness for the disease [8, 9, 10, 11, 12, 13, 14, 104, 105] causing a substantial increase their consumption by the human population [15]. According to the Brazilian Association of Distribution and Logistics of Pharmaceutical Products, the drugs present in the COVID Kit had an 857% increase in sales between April 2020 and March 2021 in Brazil [16]. Among the drugs in question, IVM presented the highest increase in sales in 2020, 557% compared to 2019. In turn, HCQ, showed an 113% increase in sales, with 963 thousand units of the drug sold in 2019 and 2 million units in 2020 [4].

It was estimated that due to the increased use of these medications during the pandemic period the release rates of them into the environment have increased significantly [106, 107]. According to Chen and coauthors [17], concentrations of AZM found in the Yangtze River, located in China, were significantly higher (up to 935 $ng.L^{-1}$), and more frequent, when compared to data from previous years, which indicated a maximus concentration of 4.3 $ng.L^{-1}$ [18]. It is important to highlight that AZM, together with other macrolide antibiotics (erythromycin and clarithromycin), was included in the European Union's watch list of emerging pollutants precisely because of its frequent detection in the aquatic environment [19].

IVM concentrations in surface water also showed a post-pandemic increase [20]. Concentrations that were previously 93 $ng.L^{-1}$ [21] were detected in the range of 1,500 $ng.L^{-1}$ in 2020 [15]. HCQ had concentrations of 95.85 $ng.L^{-1}$ detected after the pandemic [22]. Other drugs used to treat COVID-19, such as antivirals (Favipiravir, Lopinavir and Ribavirin) and dexamethasone, also showed an increase in environmental concentrations compared to previous years [20]. In Mexico City, Durán-Álvarez and coauthors [23] monitored the wastewater concentration of some drugs used during the period of the COVID-19 pandemic. High concentrations of dexamethasone, AZM and IVM were found in the wastewater treatment plant tributaries, with the latter two being detected in the range of 2,873.7 to 5,819.6 $ng.L^{-1}$ and 1,219.8 to 4,622.4 $ng.L^{-1}$, respectively. After treatment, removal rates were 74.4% for AZM and 88.1% for IVM.

Since the COVID-19 pandemic was a recent event, there is still no broad knowledge of the effects that the increase in these compounds in water can cause to the aquatic ecosystem [24]. There is a lack of data regarding the environmental toxicity of these drugs and their mixtures, as there is the possibility of a synergistic interaction between them, further increasing the toxic effects [15, 25, 107]. During preparation of this study, only 11 papers were found that dealt with the potentially adverse effects of exposing aquatic organisms to chloroquine. Of these, five were carried out in fish [26, 27, 28, 29, 30], three in *Daphnia magna* [28, 31, 22], one in the bivalve mollusk *Mytilus edulis* [32], one in *Vibrio fischeri* [28] and one in microalgae [28]. When considering only HCQ, only eight articles were found (all published from 2020 onwards), being four on *Danio rerio* [30, 33, 34, 35], one using *Physalaemus cuvieri* tadpoles [25], one in *D. magna* [22], one with the microalgae *Chlorella vulgaris* and the cyanobacteria *Synechococcus elongatus* [36], and another using mesocosmos [37]. For AZM, 40 studies were found, mostly on, such as *D. rerio* [38-42] and *Clarias gariepinus* [43, 44] and in the microcrustacean *Daphnia sp.* [45-47]. Only four studies using microalgae as bioindicators were found [36, 45, 48, 49].

In order to assess the negative impacts caused by the presence of contaminants in aquatic organisms, bioindicators are used, such as microalgae [50]. Aquatic pollutants, including PhaCs, are known to adversely affect important aspects of essential microalgae physiology, such as their growth [28,36, 70,71,96,100,103] and chlorophyl production [70, 85]. These effects are often a consequence of the interference of PhaCs on cellular physiology, which includes an exacerbation of the generation of reactive oxygen species (ROS) in microalgae, due to impaired aerobic metabolism and biotransformation reactions. As a consequence, increased levels of protein carbonyls (PC) and lipid peroxidation by-products, such as malondialdehyde (MDA) are formed, due to excessive oxidative reactions [70, 71, 109]. To counteract ROS effects, modulation of antioxidant enzymes, such as catalase (CAT) and glutathione *S*-transferase (GST) [36, 50, 77, 96] often occurs, as a defensive mechanism against cellular oxidative injuries. *Desmodesmus subspicatus* (Chlorophyta, Chlorophyceae) is a freshwater green microalga and is an example of a bioindicator largely used in toxicity tests [51, 52]. Due to the marked importance of microalgae in aquatic ecosystems, as they are the main producers of oxygen and represent the basis of the food chain for several species of animals, ecotoxicological studies on these organisms are relevant [53].

Considering the recent increase in the human consumption of the COVID kit drugs and the lack of information of their effects on aquatic organisms, in this study we aimed to evaluate how HCQ, IVM and AZM affects the growth, survival, chlorophyl levels and biochemical parameters related to oxidative stress (activity of the antioxidant enzymes catalase and glutathione S-transferase, lipid peroxidation and protein oxidation levels) of the microalgae *D. subspicatus*.

MATERIAL AND METHODS

Drugs

The drugs used in the study were hydroxychloroquine (HCQ) (CAS # 747-36-4), ivermectin (IVM) (CAS # 70288-86-7) and azithromycin (AZM) (CAS # 117772-70-0), all purchased from Sigma-Aldrich, St Louis. The drugs were diluted in distilled water, in the case of hydroxychloroquine, and in methanol (CAS # 67-56-1), in the case of the other drugs, and the solutions were stored at -20 ℃ before used.

Desmodesmus subspicatus culture

The microalgae inoculum was cultivated in Erlenmeyer flasks (250 mL), containing 100 mL of nutrient medium (CHU), prepared and autoclaved in accordance with the Brazilian standards set out in NBR 12648 - Aquatic ecotoxicology - Chronic toxicity - Test method with algae (Chlorophyceae) [54]. The microalgae cultures were maintained in a cultivation incubator (TECNAL – TE 424) with lighting (<4500 lux provided by fluorescent lamps) and controlled temperature (20 ± 2 ℃), statically and without aeration. In order to keep it in good condition, maintaining a supply of cells in the logarithmic growth phase available for carrying out toxicity tests, replicates were carried out weekly. For this, 1 mL of inoculum in exponential growth phase was transferred to a new autoclaved Erlenmeyer flask, containing 100 mL nutrient medium (CHU), also kept in a cultivation incubator under the conditions already mentioned.

After the period of initial adaptation of the inoculum to laboratory conditions, in order to guarantee the quality of the method, a calibration curve was carried out, establishing the relationship between cell count and absorbance. For this, the microalgae were cultivated for 72 hours in test medium (DIN) at a temperature between 23 and 27 ℃, with continuous lighting above 4500 lux and continuous agitation, between 100 and 175 rpm, in accordance with NBR 12648 [54], and then diluted to concentrations of 100, 50, 25, 12.5, 6.25, 3.12 and 1.56%. These dilutions were subjected to cell counting in a Neubauer chamber, using optical

microscopy (LEICA DM500) and, in the same way, their biomass was determined through spectrophotometry with absorbance reading at 410 nm (UV/VIS Spectrophotometer - SHIMADZU 1650 PC), allowing to establish a linear regression. The wavelength used was defined based on previous tests that identified the best point for reading the culture. In these tests, three cultures had their absorbance measured at different wavelengths, with the 410 nm spectrum being defined for use in the test, which presented higher absorbance values in the three situations. Based on this calibration test, an initial density (day zero) of 10^5 cells.mL^{-1} were used to expose the microalgae to the contaminant in each of the 100 mL cultures, ensuring that the culture did not exceed a total of 10^7 cells.mL^{-1} at the end of the exposure period (72 h) to the tested PhaCs.

Finally, to guarantee the quality of microalgae cultivation for PhaCs exposure tests, sensitivity tests were carried out monthly also in accordance with NBR 12648 [54], using a reference substance, potassium dichromate (25 mg.L^{-1}). To verify the sensitivity of the microalgae and calculate the effective concentration (EC50) after 72h to inhibit growth, an aliquot with a concentration of 10^5 cells.mL^{-1} was exposed to 5 concentrations of potassium dichromate solution (0.2; 0.4; 0.6; 0.8 and 1.0 mg.L^{-1}), in 100 mL of test medium (DIN). The test was carried out in triplicate, for 72 hours at a temperature between 23 and 27 ℃, with continuous lighting above 4500 lux and agitation, between 100 and 175 rpm, on an orbital shaking table (Lucadema, TECNAL).

At time zero, the three controls and a replicate of the highest and lowest concentration had their pH (TECNAL) and absorbance determined at 410 nm. After 72 hours of exposure, the pH of the same cultures was determined, as well as the absorbance of all concentrations and replicates. The tests were considered valid in situations where there is no variation greater than 1.5 in the pH of the controls and the microalgae growth is greater than 16 times.

Toxicity tests using environmental concentrations of the COVID kit drugs

The toxicity tests with hydroxychloroquine, ivermectin and azithromycin, were carried out under the same conditions and with the same method used for the sensitivity tests described previously. Since the drugs ivermectin and azithromycin have low solubility in water, it was necessary to use methanol or dimethyl sulfoxide (DMSO), respectively, to dilute them. Therefore, the stock solutions were prepared so that the amount pipetted into the algal culture, at the beginning of the experiment, was as small as possible, 10 µL (0.01%). As ivermectin was only soluble in methanol, when testing the effects of the mixture of the compounds, methanol was used as vehicle to dilute all compounds. To be aware of the changes caused by the solvents, we checked the effects of methanol and DMSO on algae growth. Methanol even at a volume of 0.01% caused a significant inhibition of *D. subspicatus* growth (average of 25.8 % from five replicates, data not shown), when compared to the growth of organisms in absence of the solvent. DMSO did not show any significant inhibition of microalgae growth. For this reason, control groups (in triplicate) also received 10 µL of the respective vehicle used for each compound: one control for hydroxychloroquine without solvent, one control for ivermectin with methanol, and one control for azithromycin with DMSO. Results from the group exposed to the mixture of compounds were compared to the control group with methanol.

Toxicity tests were done using environmentally realistic concentrations of each compound, based on data previously reported in the scientific literature. Hydroxychloroquine concentrations ranging from 13 to 420 µg.L^{-1} have been reported in river water, in Spain [55] and in wastewater treatment plants, in Northern Italy, at concentrations of 1.77 µg.L^{-1} [56]. For ivermectin, concentrations of 1.8 µg.L^{-1} have already been reported in South Africa in surface water samples [57] and concentrations of 3.2 µg.L^{-1} were found in rivers in the city of Curitiba, Brazil [36], while azithromycin was found at concentrations ranging from 0.01 to 3.4 µg.L^{-1} in Sava river, in the Republic of Croatia [58] and in wastewater treatment plants at concentrations ranging from 0.12 to 14.8 µg.L^{-1} in the United States of America [59]. Thus, in our study the microalgae were exposed to the compounds alone or in mixture, at concentrations of 1.0, 5.0, 10.0, 50.0 and 100.0 µg.L^{-1}, in triplicate, for 72 h. The mixtures were prepared using the same concentrations for each drug.

Determination of EC50 values for the COVID kit drugs

Tests to determine the effective concentration (EC50) capable to inhibit the growth of the microalgae were also performed, similar to the methods described by Marques and coauthors [36]. The algae were initially exposed for a period of 72 h to the drugs in isolation at concentrations of 0, 200, 400, 600, 800 and 1000 µg.L^{-1}. Based on this preliminary test, changes in drug concentrations were necessary to betted determine the EC50, except for hydroxychloroquine. For ivermectin concentrations of 0, 100, 200, 400, 600 and 800 µg.L^{-1} were used due to difficulty of diluting it in solvents. For azithromycin, exposure was performed using 0, 25, 50, 75, 100, 125, 150 and 175 µg.L^{-1} so that the EC50 could be determined. Same solvents used to dilute the drugs in toxicity tests were used in tests to determine EC50 values, and all concentrations used

were added to the aquaria in a final volume of 10 μL (0.01%). The groups without the drugs (0 $\mu g.L^{-1}$) received only 10 μL of the respective solvent. To check growth rate during the test, an aliquot of 1 mL of algal culture was collected after 72 hours of exposure to the respective drug and had its absorbance determined at 410 nm. The absorbance was used to calculate algae biomass in the culture medium, and this data was used to determine growth inhibition, using the formulae: Inhibition (%) 1=(Bc-Bt)/Bc ×100, where Bc and Bt represent the biomass concentrations in the control and treatment, respectively.

Chlorophyll a and b determination

Chlorophyll a and b content was quantified using the method described by Kurade and coauthors [60] with adaptations. A 10 mL aliquot of the algal culture was centrifuged at 3,075 g (HERMLE Z 326 K) for 30 minutes. After centrifugation, the supernatant was discarded and 10 mL of 80% methanol was added to resuspend the precipitate, which was then heated in a water bath at 60 ℃ for 10 minutes, and centrifuged again at 3,075 g for 10 minutes. At the end of this process, the absorbance of the supernatant was analyzed at wavelengths 665 and 652. The values obtained were used to calculate the chlorophyll a and b contents according to Wellburn [61] and Gomaa and coauthors [50].

Biochemical biomarkers

To determine the activity of antioxidant enzymes, at the end of 72 hours of exposure of microalgae to the drugs, a 50 mL aliquot of the culture was collected and centrifuged at 3,075 g (HERMLE Z 326 K) for 30 minutes. The precipitate formed at the bottom of the tube was stored in 200 μL of sodium phosphate buffer (50 mM, pH 7.5) at – 60 ℃.

Subsequently, the cells were disrupted by homogenization with glass beads (2 mm) [62] The process was carried out using a vortex (KASVI K45-2820), where the sample remained for 30 minutes, in a cooled environment (4 ℃). The material obtained was aliquoted and stored at a temperature of – 60 ℃ until enzymatic analyzes were carried out. The supernatant of the samples was used for the analysis of the antioxidant enzymes catalase (CAT), glutathione-S-transferase (GST) as well as for the analysis of protein oxidative damage by quantifying the content of carbonyl groups and lipid peroxidation levels.

CAT activity was determined using the method proposed by Matsumura and coauthors [63] 150 μL of the sample was added to 750 μL of sodium phosphate buffer (50mM, pH 7.5) and 100 μL of a hydrogen peroxide solution (200mM). The activity of the enzyme was estimated by monitoring the consumption of H2O2 by the enzyme at 240 nm (UV/VIS Spectrophotometer - SHIMADZU 1650 PC) for 60 seconds. GST activity was determined by the method of Habig and Pubst [64], using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. 40 μL of the sample was added to 150 μL of potassium phosphate buffer (133 mM pH 6.5), followed by 20 μL of GSH (50 mM) diluted in sodium phosphate buffer (100 mM pH 7.6) and 10 μL of CDNB (20 mM) diluted in absolute ethanol. The production of CDNB-SG conjugate was monitored at 340 nm for 5 minutes in a microplate reader (FLUOstar Omega BMG LABTECH). Total protein content was determined using the Bradford method [65] in all samples.

To access the status of oxidative damage to proteins in the algae, protein carbonyl (PC) levels were analyzed according to the method proposed by Levine and coauthors [66], with adaptations. 200 μL of the sample was added to reaction medium containing 200 μL 2,4-dinitrophenylhydrazine (DNPH) (10 mM) diluted in hydrochloric acid (2M) and kept in a water bath for 1 hour, shaking the samples every 15 minutes. 280 μL of 28% trichloroacetic acid were added to the mixture, which was subjected to centrifugation (10,000 g for 10 min). The precipitate formed was resuspended in 250 μL of sodium dodecyl sulfate (6%) and centrifuged again (10,000 g for 10 min). The absorbance of the supernatant was read at 370 nm in a microplate reader. Results are expressed in nmol.mg protein-1.

To determine lipid peroxidation levels, another 30 mL aliquot of the culture was centrifuged at 3,075 g for 30 min. The precipitate was stored in sodium phosphate buffer (50 mM, pH 7.5) at -60 ℃ until analysis. Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels according to the method proposed by Almeida and coauthors [67], with modifications. The sample was shaken for 30 minutes with glass beads (2 mm) in a vortex (KASVI K45-2820) in a cooled environment (4 ℃). Subsequently, 300 μL of 0.4% thiobarbituric acid (TBA, Sigma-Aldrich, Germany) diluted in HCl (0.2 M) were added to 300 μL of the homogenized sample. The mixture was heated to 90 ℃ for 40 min, and subjected to extraction with 500 μL of n-butanol (Sigma-Aldrich, USA) followed by centrifugation (5,000 g for 3 min). The supernatant was read at 540 nm on a microplate reader (FLUOstar Omega BMG LABTECH). MDA was quantified based in a tetramethoxypropane standard of curve prepared using the same procedure as for samples. Results are expressed in nmol.mg protein-1.

Statistical analyses

The data obtained in pigment quantification and biochemical analyzes were tested for normality using the Shapiro-Wilk test and for homogeneity using the Bartlett test. Differences between treatment groups and solvent control were statistically analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. In the case of non-parametric data, the Kruskal-Wallis test was used followed by the Dunn test. GraphPad Prism® software (version 8.0, GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. This software was also used to determine the EC50(72h) (concentrations of the substance in which there was 50% inhibition of growth rate) for concentration-dependent relationships, using a non-linear regression curve fitting model. All tests mentioned had a significance level set at $p<0.05$.)

RESULTS

Effects of environmentally relevant concentrations of the COVID kit drugs on algae growth

Both IVM and HCQ did not affect *D. subspicatus* growth in none of the concentrations tested. In contrary, the highest concentration of AZM and MIX caused a significant inhibition (35.3 and 49.8%, respectively) of algae growth (Figure 1).

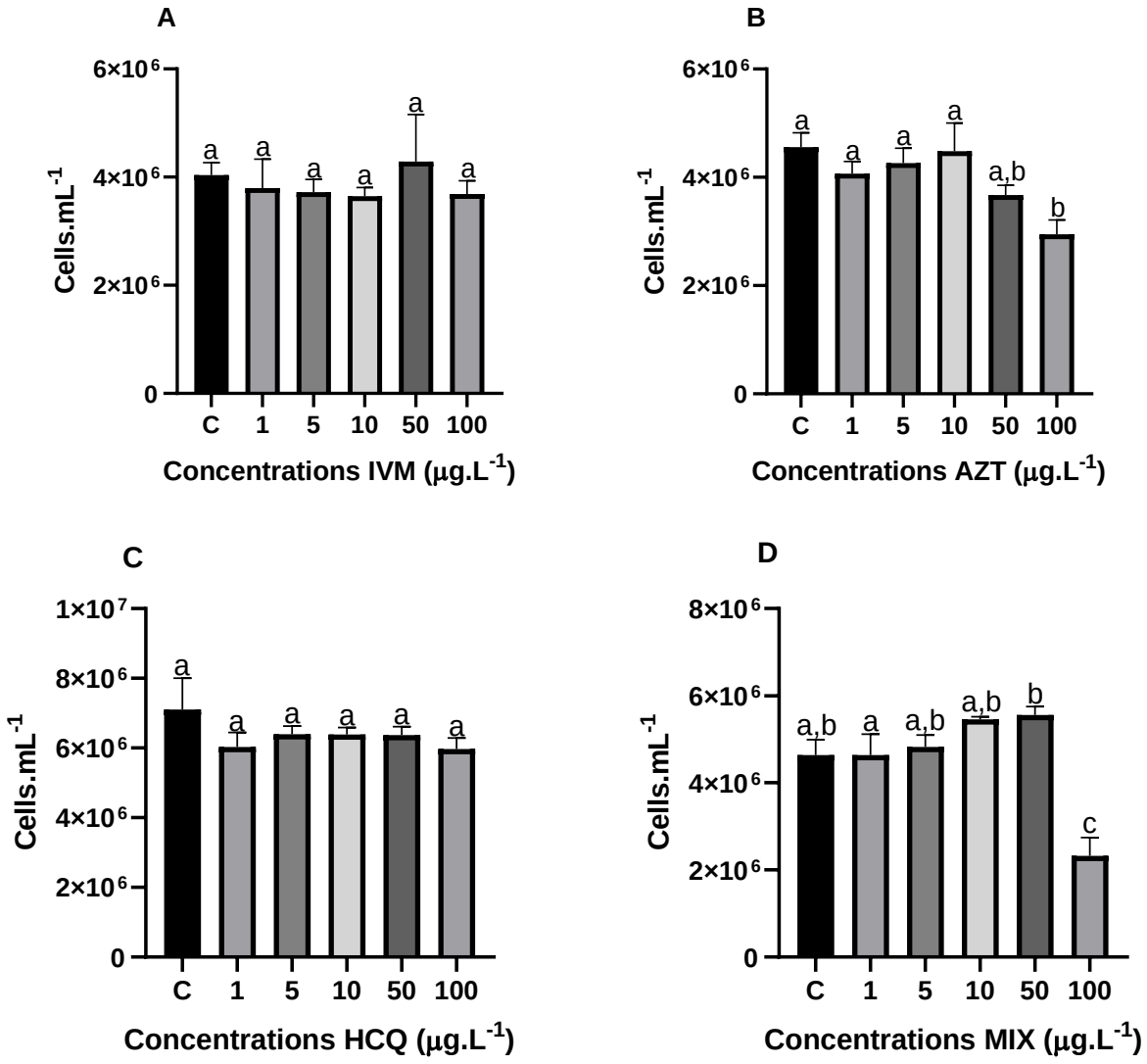


Figure 1. Number of *D. subspicatus* cells in 1 mL of culture medium after 72 h of exposure to the drugs IVM, AZM, HCQ and MIX, at concentrations of 0 (C – control) 1, 5, 10, 50 and 100 µg.L-1. Values are represented as the mean ± standard deviation. Different letters indicate significant difference ($p<0.05$) using the Tukey test.

Effects of HCQ, AZM and IVM on algae growth and determination of EC50 (72h) values.

The estimated EC50 (72h) values of HCQ and AZM were 510 and 80 μg.L^{-1}, respectively. Due to its low solubility at high concentrations, it was not possible to estimate the exact value of EC50 (72h) for IVM, which was higher than 800 μg.L^{-1}.

Chlorophyl a and b levels

Levels of chlorophyl a and b did not change in none of cultures treated with drugs in isolation in comparison to the control group (Figure 2). However, cultures exposed to the highest AZM concentration presented lower levels of both chlorophyl than the cultures exposed to 5 and 10 μg.L^{-1} of same compound.

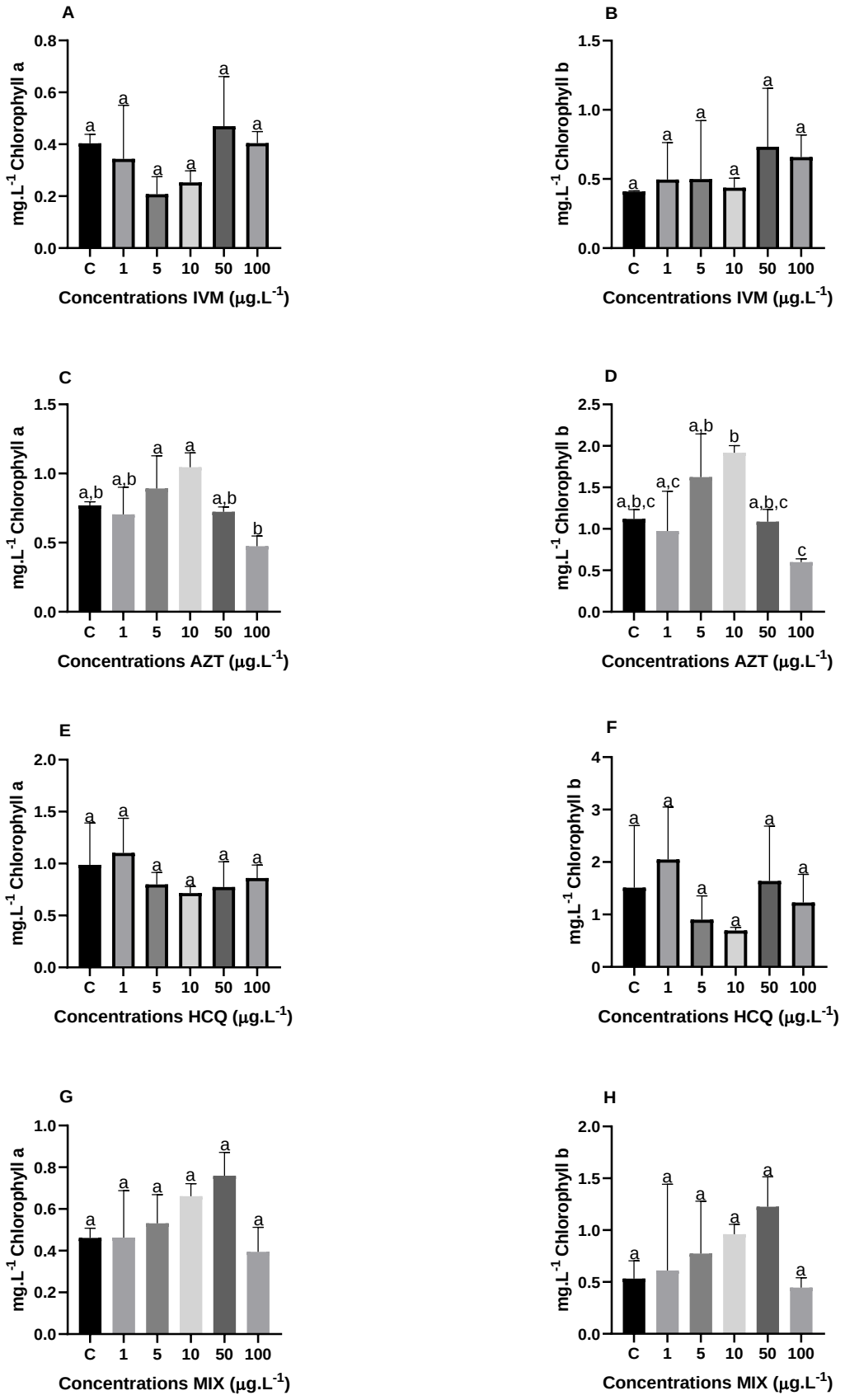


Figure 2. Chlorophyll a and chlorophyll b pigment content of *D. subspicatus* after 72h of exposure to the drugs IVM, AZM, HCQ and MIX, at concentrations of 0 (C – control) 1, 5, 10, 50 and 100 µg.L^{-1}. Values are represented as the mean ± standard deviation. Different letters indicate significant difference ($p<0.05$) using the Tukey (A, B, C, D, E, F, G) or Dunn (H) test.

Biochemical markers

The microalgae cultures that were exposed to IVM (Figure 3A) and to the mix (Figure 3D) did not show significant differences in CAT activity. Exposure to 50 and 100 µg.L^{-1} AZM resulted in significant lower CAT activity in comparison to the groups that were exposed to 1 and 5 µg.L-1 (Figure 3B). Moreover, CAT activity was higher than control in algae exposed to 5, 10 and 100 µg.L^{-1} of HCQ (Figure 3C). GST activity was not significantly altered by none of the treatments, when compared to control values. However, algae cultures that were exposed to the highest concentration of the MIX showed lower GST activity than those exposed to 5 of the MIX.

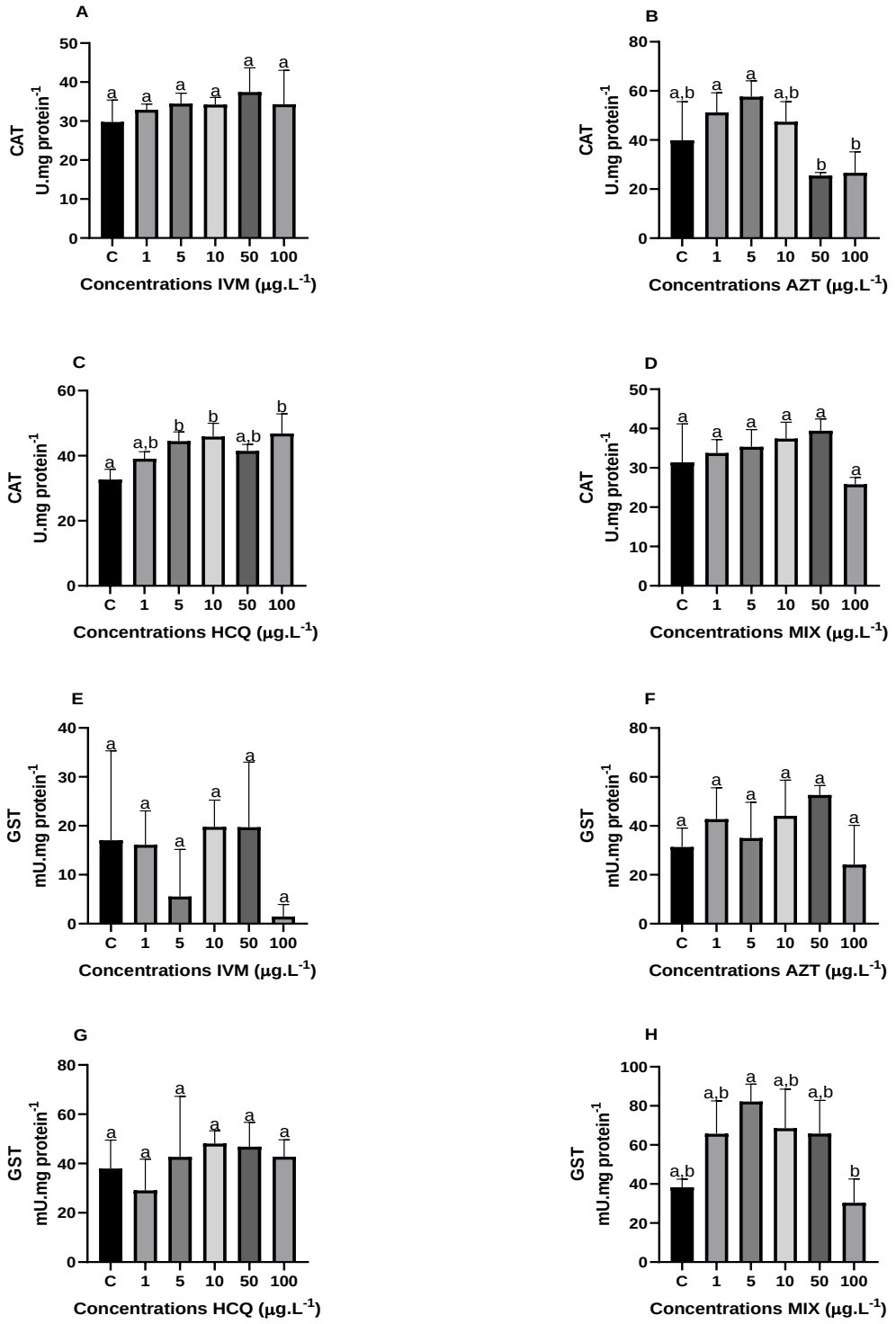


Figure 3. Activity of CAT and GST enzymes of *D. subspicatus* after 72h of exposure to the drugs IVM, AZM, HCQ and MIX, at concentrations of 0 (C – control) 1, 5, 10, 50 and 100 µg.L^{-1}. Values are represented as the mean ± standard deviation. Different letters indicate significant difference ($p<0.05$) using the Tukey (A, B, C, F, G, H) or Dunn (D, E) test.

MDA levels did not show significant differences between any of the treatments (Figure 4). With regard to the levels of protein carbonyls (PC), no differences were observed when comparing the control group with the cultures exposed to the drugs. However, *D. subspicatus* culture exposed to the highest concentration of the mix presented higher PC levels than the culture at 50 µg.L^{-1}.

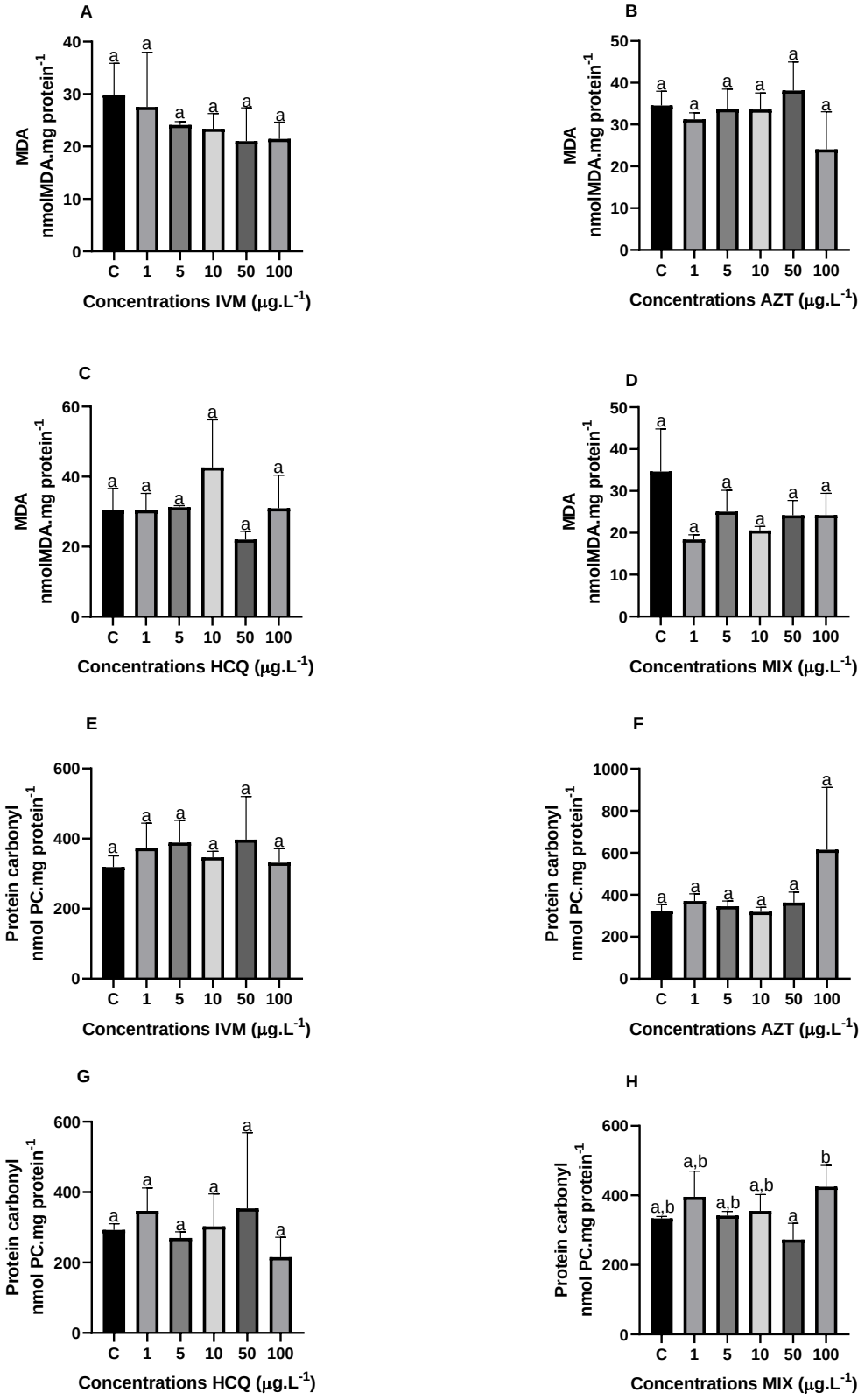


Figure 4. Levels of MDA and carbonyl proteins of *D. subspicatus* after 72h of exposure to the drugs IVM, AZM, HCQ and MIX, at concentrations of 0 (C – control) 1, 5, 10, 50 and 100 µg.L-1. Values are represented as the mean ± standard deviation. Different letters indicate significant difference ($p<0.05$) using the Tukey (A, B, C, E, H) or Dunn (D, F, G) test.

DISCUSSION

Toxicity tests

IVM did not exert substantial toxicity to *D. subspicatus*, showing no growth inhibition at tested concentrations. This result agrees with Martins [97], which also did not observe any effects of IVM on *D. subspicatus* growth. The EC50 test also predicted concentrations greater than 800 $\mu g.L^{-1}$ to cause any effect on growth. These results agree with EC50 values found by Garric and coauthors [45] for the microalgae *P. subcapitata*, which was greater than 4000 $\mu g.L^{-1}$. For abamectin, a compound whose structure is very similar to IVM, Tišler and Eržen [68] found an EC50 of 4400 $\mu g.L^{-1}$ (72 h) for the microalgae *S. subspicatus*, in 72 hours tests, while a value of 153,730 µg.L-1 was estimated by [36] for *Chlorella vulgaris*. Similarly, Kołodziejska and coauthors [98] also reported very low toxicity of doramectin, another antiparasitic drug, for the green algae *Scenedesmus vacuolatus*. For *Daphnia sp.* the EC50 concentrations of IVM (5.7 $ng.L^{-1}$; [45]) or abamectin (5.54 µg.L-1; [48]) were significantly lower during 48 hours of exposure, evidencing the lower IVM toxicity for microalgae, including *D. subspicatus*. The greater toxicity reported for microcrustaceans is probably due to the mechanism of action of IVM, as it interacts with chlorine channels allowing a greater influx of the ion and culminating in nervous hyperpolarization [69].

AZM proved to be considerably more toxic to *D. subspicatus* than IVM, decreasing algae growth with an EC50 of 80 $\mu g.L^{-1}$. Similarly, Mao and coauthors [70] reported an EC50 (96 h) of 41.6 $\mu g.L^{-1}$, for the microalgae *C. pyrenoidosa*, while Almeida and coauthors [71] found an EC50 (72 h) of 51 $\mu g.L^{-1}$ for *Raphidocelis subcapitata*. Guo and coauthors [96], also showed that the macrolide antibiotic clarithromycin was able to inhibit the growth of algae *Raphidocelis subcapitata* and *C. vulgaris*, at $\mu g.L^{-1}$ concentrations. When comparing the toxicity of 13 different antibiotics to the freshwater green algae *Pseudokirchneriella subcapitata*, AZM presented the highest toxicity [100]. For other organisms, such as *Daphnia* sp., AZM is often less toxic, having EC50 values ranging from 100 to 150 $mg.L^{-1}$. [72, 46, 73]. For *V. fischeri*, a considerably lower EC50 was reported, when compared to *D. subspicatus* (30.26 x 10-5 $mol.L^{-1}$; [74], demonstrating greater resistance of the microalgae to this antibiotic. In contrast, Harada and coauthors [103] noted a higher toxicity of AZM to algae than to bacteria. The action mechanism of AZM consists on the inhibition of bacterial protein synthesis through its binding to the 50S ribosomal subunit, acting as a bacteriostatic agent, inhibiting bacterial growth [75, 76]. AZM was also shown to weaken the photosynthetic activities of algae, to reduce the energy reserves and to alter the cellular structure [70]. Other macrolide antibiotics, such as erythromycin, roxithromycin, have already had reported toxic effects on microalgae, such as genotoxicity, interfering with DNA repair genes [77], and inhibiting the chloroplast gene translation process, which is involved in the synthesis of several proteins important for photosynthesis [78]. These mechanisms may explain the azithromycin toxicity observed in *D. subspicatus*. It is important to highlight that AZM is often found in anthropized aquatic environments at concentrations higher than predicted no-effect concentration for microalgae [99, 101, 102], especially after the COVID19 pandemic.

HCQ presented an EC50 of 510 $\mu g.L^{-1}$ for *D. subspicatus*, which was much lower than values found for the microalgae *C. vulgaris* by Marques and coauthors [36] (200,160 $\mu g.L^{-1}$; 72 h). These authors also tested HCQ toxicity for the cyanobacterium *S. elongatus*, finding an EC50 (72 h) of 6.71 $\mu g.L^{-1}$, evidencing the higher sensitivity of the bacteria to HCQ. For the analogous compound chloroquine, Zurita and coauthors [28] also found relative high values of EC50 (48 h) for the microalgae *C. vulgaris* (27 $mg.L^{-1}$), for *D. magna* and (9 $mg.L^{-1}$), and for *V. fischeri* (126 $mg.L^{-1}$). Comparatively, our results demonstrate the great sensitivity of *D. subspicatus* to HCQ. The mechanism of action of HCQ is broad and dependent on the context associated with its use. The drug mainly interferes with lysosomes, increasing the pH inside them and inhibiting the action of their enzymes [79]. The alkalinization effect has also been observed inside cells, in acidic organelles, limiting the uptake of iron and interfering with infection by intracellular microorganisms, such as bacteria and fungi [80], which may be related to its toxicity to microalgae [36]. Furthermore, there is evidence of a strong interaction between HCQ and DNA [81].

Regarding the mixture with the three drugs, there was considerable growth inhibition in cultures exposed to the highest concentration. It is possible that this inhibition is related only to the effect of AZM on microalgae, since the EC50 found in this study for the drug was 80 $\mu g.L^{-1}$. However, any potential synergistic effects of the other compounds in the mixture should not be disregarded. Agreeing with this, Marques and coauthors [36] noted interactive effect of mixtures of IVM, AZM and HCQ on growth, photosynthesis and antioxidant activity of *C. vulgaris*. Similarly, Arreguin-Rebolledo and coauthors [108] also reported important synergistic toxic effects of AZM and IVM mixture in the freshwater rotifer *Lecane papuana*.

Effects on biochemical parameters

With regard to biochemical analyses, only the activity of CAT in cultures exposed to HCQ showed an increase at concentrations of 5, 10 and 100 $\mu g.L^{-1}$, in comparison to the control group. This effect may indicate a microalgae response to increased cellular production of H_2O_2 due to intoxication [82. 83]. The absence of changes in biomarkers of cellular injury (MDA and PC) may indicate success in neutralizing these reactive species by catalase, preventing protein carbonylation and lipid peroxidation [84]. The lack of CAT increase at the lowest concentration (1 $\mu g.L^{-1}$) is consistent with results found by Marques and coauthors [36], in which no change in CAT activity was observed in *C. vulgaris* after exposure to 2 $\mu g.L^{-1}$ of HCQ. However, the lack of similar CAT increase in microalgae exposed to the drug mixture is intriguing, since the mixtures contained the same HCQ concentrations. Possible antagonistic effects caused by the other chemicals in the mixture could explain this result. It should be noted that despite AZM exposure did not affect CAT activity, this enzyme was significantly lower in cultures exposed to 50 and 100 $\mu g.L^{-1}$, in comparison to those exposed to 1 and 5 $\mu g.L^{-1}$. A possible inhibitory effect of AZM on CAT would be then masking or suppressing the HCQ induction effect. Although most studies regarding the effects of antibiotics such as clarithromycin [77, 96] ciprofloxacin, tetracycline and amoxicillin [50] on microalgae show an inducing effect on CAT, Marques and coauthors [36] observed an inhibitory effect of AZM on CAT activity of *C. vulgaris*, which is consistent with our hypothesis.

Despite evidences that sulfonamide antibiotics are capable of inhibiting chlorophyll synthesis by interfering with gene expression of the chloroplasts [85], none of the drugs altered the content of chlorophyll a and b. This finding also contrasts with the lower chlorophyll a and b content in *C. pyrenoidosa* after AZM exposure [70], suggesting *D. subspicatus* is more resistant to this AZM effect. To the best of our knowledge, studies regarding the effects of IVM and HCQ on algae chlorophyll levels are absent in the scientific literature. Nevertheless, Sanderson and coauthors [86] showed that IVM added to outdoor aquatic mesocosms didn't affect chlorophyll a level.

Levels of cellular injury biomarkers (MDA and PC) and GST activity were also not affected by the drugs. Contrasting to our results, AZM exposure increased ROS generation and MDA levels in *C. pyrenoidosa* and *R. subcapitata* [70, 71], also suggest that *D. subspicatus* is more resistant to toxic AZM effects in comparison to other species. Studies on the effects of IVM and HCQ on PC levels and GST activity in algae are absent in the scientific literature.

Discrepancies in responses to contaminants between different algae species are possibly due to their capacity of metabolize different chemicals. Solovchenko and coauthors [87] reported that *Desmodesmus* sp. displays a relatively high tolerance to commercial formulations of pharmaceutical products (azithromycin, ibuprofen, ceftriaxone, diclofenac, amoxicillin + clavulanate potassium). Concentrations of 3, 300 and 1000 $\mu g.L^{-1}$, of practically all tested contaminants, had a stimulating effect on the production of chlorophyll and carotenoids in microalgae, and only azithromycin at the highest concentration, significantly reduced the pigment content. According to the author, these results were possible due to the ability of certain species of microalgae to use pharmaceutical compounds as a carbon source.

Another explanation for the resistance of *D. subspicatus* to contaminants is the structure of the cell wall. This microalgae species has a fourth layer of sporopollenin [88, 89, 90], a biopolymer that provides resistance to the cell wall, protecting the organism from environmental stress, desiccation and UV radiation [91, 92]. Thus, there is the possibility that this additional layer provides greater resistance to the toxicity of chemical compounds to the species, compared to other species. The presence of sporopollenin in the cell walls of different microalgae species is varied. The subgenus Scenedesmus has three layers of the biopolymer in its structure [88], one layer less than *D. subspicatus*. The presence of sporopollenin in the cell wall of *C. vulgaris* is still debated [93]. It is suggested that it is present in a layer, distributed in unknown quantities [94]. Species such as *C. fusca* and *C. sorokiniana* do not have the biopolymer in their structure [95]. Therefore, we hypothesize that the structure and thickness of the cell wall would be an important factor affecting pollutant toxic effects to algae, and further studies on this aspect are important for clarification.

CONCLUSION

Given the results presented, it was possible to establish the EC50 for the drugs IVM, AZM and HCQ for *D. subspicatus*, a microalgae species commonly used in standardized ecotoxicological tests. In general, IVM and HCQ were less toxic than AZM, as evidenced by the lower EC50 value estimated for AZM and the significant growth inhibition of algae culture at the highest AZM and MIX concentrations. However, effects at subcellular levels showed an increase of CAT in algae exposed to HCQ, which would represent a higher stressful cellular condition due to increased production of H_2O_2. According to European Regulation, substances that have an EC50, for microalgae, lower than 1 $mg.L^{-1}$ are considered very toxic to the aquatic environment, therefore, both AZM and HCQ fit in this classification. Taking together, our results points to

potential negative effects of the COVID-19 kit to freshwater algae, especially if considering that the effects reported here occurred in short-term exposure experiments (72 h). More intense effects could be predicted if prolonged exposure periods are considered, which would imply in important ecological negative impacts.

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