

TOXIC EFFECTS OF SULFAMETHOXAZOLE (SMX) ON A TROPICAL FRESHWATER MICROALGA, *CHLORELLA* SP.

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Abstract: This study investigates the acute and sub-chronic toxicity of the emerging antibiotic, sulfamethoxazole (SMX) on the tropical freshwater microalga *Chlorella* sp. under laboratory condition. An acute exposure for 96 h and a sub-chronic exposure for 10 days were conducted to explore the adverse effects, utilizing growth inhibition, pigment content, and cell diameter as endpoints. The calculated half maximal (50 %) effective concentration (EC₅₀) values for SMX after 24 h and 96 h were estimated to be 0.56 mg/L and 0.41 mg/L, respectively. Sub-chronic exposure unveiled a pronounced inhibitory effect of SMX on algal growth, with significant and dose-dependent increases in growth inhibition observed. Across all treatments, exposure to SMX resulted in growth inhibition and a decline in chlorophyll-a (Chl-*a*) concentration in the test algae. Notably, at a concentration of 1 mg/L, SMX completely inhibited the growth of *Chlorella* sp. and reduced Chl-*a* content by up to 98 %. High concentrations of SMX (0.5 and 1.0 mg/L) led to a decrease in cell diameter in the tested algae. This study contributes valuable insights into the acute and sub-chronic toxic effects of environmentally relevant concentrations of antibiotics on tropical freshwater microalgae.

Keywords: antibiotics, acute exposure, sub-chronic toxicity, growth inhibition, cell diameter

1. INTRODUCTION

Pharmaceuticals have long been used to treat or prevent infective diseases in human as well as in animals (Kovalakova et al., 2020). They also have been used in large amounts in agriculture to protect plant growth and in livestock and aquaculture (Carvalho & Santos, 2016). Antibiotics are one of the pharmaceutical groups that has been used with increasing trend in the last two decades (Klein et al., 2024). Consequently, the extensive utilization of antibiotics has led to an increase in their concentration in the aquatic environment (Bielen et al., 2017; Maghsodian et al., 2022). Different kinds of antibiotics have been detected in wastewater treatment plants, surface water, and various sources of drinking water (Liu et al., 2018; Zhao et al., 2021; Yang et al., 2021).

The sulfonamide antibiotic sulfamethoxazole (SMX) is a bacteriostatic broad-spectrum antibiotic

commonly employed against infectious bacteria in both humans and animals (Duan et al., 2022; Xu et al., 2022). Among sulfonamides, sulfamethazine (SMZ) and SMX are prevalent in various environmental sources, including wastewater, freshwater, groundwater, soil, as well as in drinking water and milk (Duan et al., 2022), while SMX is listed in the top six most prescribed antibiotic (Xu et al., 2022). Sulfonamide antibiotics are the main pollutant in the surface waters of many countries throughout the world (Ray et al., 2018; Xu et al., 2022). It is estimated that over 10 tons of SMX are discharged annually from the Mekong River Basin into the seas (Shimizu et al., 2013). The reported concentration of SMX in certain surface waters in Vietnam was 1720 ng/L (Shimizu et al., 2013).

Antibiotics have been developed to eliminate bacteria, protecting both humans and animals (Wang et al., 2016). However, only a small portion of

antibiotics undergoes metabolism in humans or animals, with the majority (70 - 90 %) being excreted unchanged through feces and urine (Maghsodian et al., 2022). Subsequently, the presence of antibiotic residues entering aquatic ecosystems has led to toxic effects on various groups of aquatic organisms. Furthermore, the prolonged existence of antibiotics in the environment can potentially pose negative impacts on human health, either directly through water consumption or indirectly through the consumption of food contaminated with antibiotics (Wang et al., 2016). SMX has been categorized by the World Health Organization (WHO) as a group 3 carcinogen (WHO, 2018), drawing increased attention to the associated adverse effects and health hazards. Prior studies have documented that SMX has induced toxic effects on various organisms, encompassing microalgae, crustaceans, amphibians, bivalves, and fish (Limbu et al., 2018; Zhou et al., 2018; Serra-Compte et al., 2019; Liu et al., 2020; Zhao et al., 2021; Xu et al., 2022; Zhang et al., 2023).

Microalgae serve as a primary producer in aquatic environment, contributing significantly in nutrient cycling to higher trophic levels (Pham, 2019; Sathasivam et al., 2019). They have been used as biological indicator of water quality and environmental health (Pham et al., 2022). Therefore, the assessment of aquatic ecosystems' quality relies on key indicators such as algal diversity and productivity.

Earlier investigations have indicated that the cytotoxicity of the majority of antibiotics to microalgae primarily stems from their interactions with organelles and biomacromolecules (Xiong et al., 2019; Xu et al., 2022). To counteract the toxic effects, algal cells initiate a series of protective responses to preserve the normal functioning of the cells. However, under highly toxic conditions, various detrimental effects may occur, such as abnormal growth, stimulation of biological macromolecules, organelle destruction, and alterations in energy balance. In severe cases, cell death has been observed in microalga cells (Xu et al., 2022).

Chlorella sp. is a unicellular green microalga that plays a crucial role in freshwater aquatic ecosystems. It is commonly found in both fresh and brackish waters (Borecka et al., 2016; Sathasivam et al., 2019). Due to its rapid reproduction and sensitivity to pollutants, *Chlorella* is widely employed as a model organism in environmental toxicology to assess the toxicity levels caused by hazardous substances. Earlier studies predominantly concentrated on the toxic effects of SMX on *Chlorella* sp. using temperate or sub-tropical isolates derived from seawater (Zhang et al., 2014; Yu et al.,

2023). Limited information is available on the biological effects of SMX on tropical freshwater microalgae, particularly the adverse effects at environmentally relevant concentrations of SMX. Thus, this study aims to examine the acute and sub-chronic toxicity of SMX on the freshwater green microalgae *Chlorella* sp. It is valuable to understand the toxic mechanisms and its potential ecological implications.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals utilized in this study were of HPLC grade. Specifically, SMX, with a purity of 99.0 %, was procured from Sigma-Aldrich (Louis, MO, USA). A comprehensive presentation of the physicochemical properties of SMX is shown in Table 1. A stock solution of SMX at 200 mg/L was prepared and stored at 4 °C before the test.

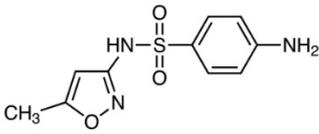
2.2. Algal culture

The green microalgae, *Chlorella* sp. (Figure 1) was originally isolated from the Tri An Reservoir in Dong Nai province, Vietnam. Isolation of *Chlorella* sp. was performed on solid agarose plates containing 1.5 % agar in COMBO medium. Raw water drops were placed on the plates and spread using a sterile inoculation loop. After several weeks, the plates with colonies were examined under a microscope to confirm the presence of *Chlorella* sp. colonies. The identified colonies were then isolated and cultured in freshwater COMBO medium with a pH of 7.5 (Kilham et al., 1998). The cultivation of *Chlorella* sp. was upheld in an incubator at a temperature of $27 \pm 1^\circ\text{C}$, with a light intensity of approximately $50 \mu\text{mol photons/m}^2/\text{s}$, following a 12-hour light and 12-hour dark cycle. For subsequent experiments, the *Chlorella* sp. stock culture, collected during the stationary phase, was employed.

2.3. Algae growth inhibition test

In a previous investigation conducted by Zhang et al. (2021), it was established that the EC_{50} value for SMX with respect to the green microalga *Raphidocelis subcapitata* (*R. subcapitata*) amounted to 0.49 mg/L after 96 h of exposure. In the current study, we delved into the impact of various SMX concentrations on the algal species. The experiment involved exposing living cells of *Chlorella* sp. to varying concentrations of SMX. In brief, the *Chlorella* sp. stock culture, collected at the stationary phase, was diluted in 100-milliliter Erlenmeyer flasks, each

Table 1. The physicochemical properties of SMX (Scholar, 2007)

Properties	Sulfamethoxazole (SMX)
Chemical structure	
Molecular formula	C ₁₀ H ₁₁ N ₃ O ₃ S
Molecular mass	253.279
Water solubility	0.61 mg/mL
logKow	0.89
pKa	1.6; 5.7

containing 50 mL of COMBO medium. The microalgae cells were initiated at a starting concentration of 4.5×10^4 cells/mL. The SMX stock solution was then added to achieve concentrations of 0, 0.05, 0.1, 0.2, 0.5, and 1.0 mg/L. Each concentration underwent three replicates, and all treatments were subsequently incubated under the previously mentioned culture conditions. To prevent cell settling, the test flasks were gently shaken once daily.

The cell density of *Chlorella* sp. was quantified daily using a Neubauer improved cell counting chamber. The concentration of SMX led to a 50 % inhibition of algal growth over 24 hours, and 96 h (referred to as EC₅₀-24h and EC₅₀-96h) was

calculated based on the SMX concentration and the algae density. The growth rate (GR) for each time period was calculated using the following equation:

$$GR_{i-j} = \frac{\ln C_j - \ln C_i}{t_j - t_i} \quad (1)$$

where GR_{i-j} represents the growth rate from time i to time j , with t_i being the initial time and t_j being the later time of exposure. C_i denotes the cell density at time i , while C_j corresponds to the cell density at time j .

The inhibition rate induced by SMX on *Chlorella* sp. was computed as follows:

$$\%IG = \frac{GR_C - GR_T}{GR_C} \times 100 \quad (2)$$

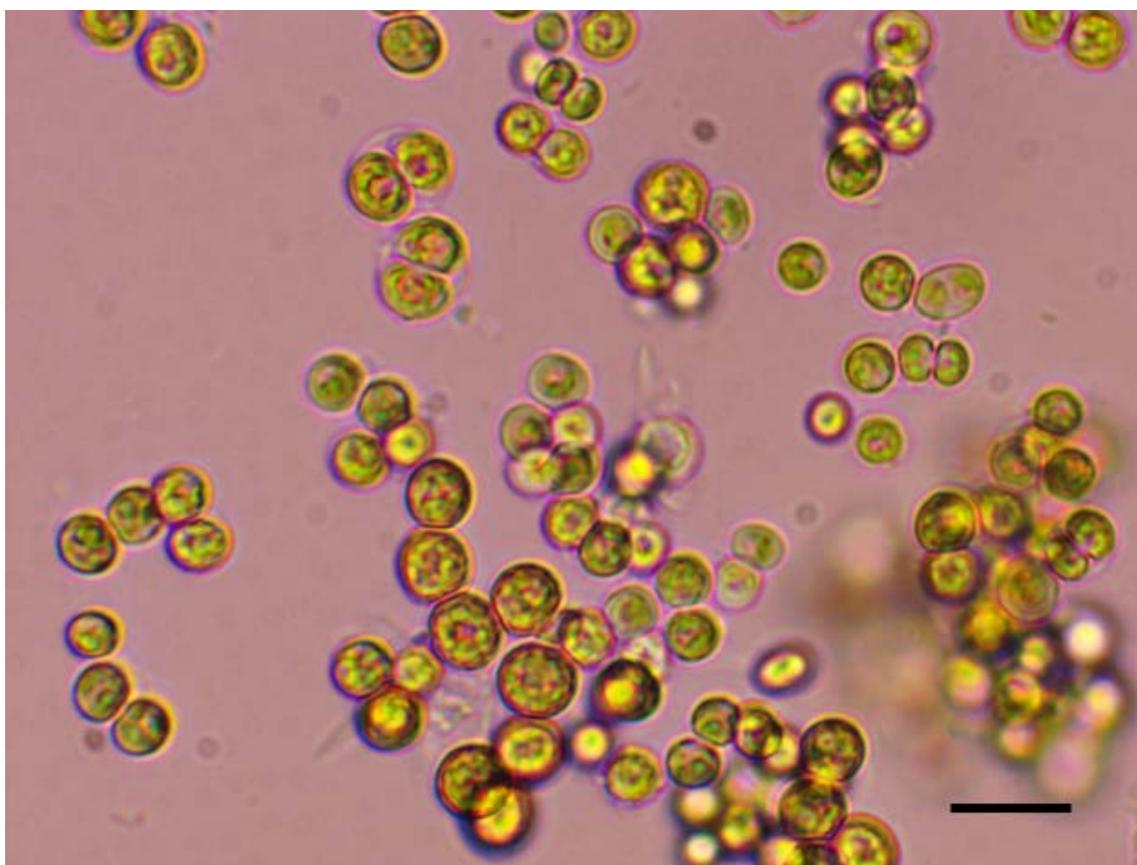


Figure 1. Microscopic images of *Chlorella* sp. in COMBO medium. Scale bar: 10 μ m.

where %IG is the growth inhibition of the *Chlorella* sp.; GR_C is the growth rate (GR) in the control group and GR_T is the growth rate for the exposure.

2.4. Sub-chronic exposure test

The sub-chronic exposure persisted for a duration of 10 days under the identical exposure conditions as those mentioned in the acute test described earlier. During the sub-chronic tests, assessments of algae density and chlorophyll-a (Chl-*a*) content were determined at the end of the experiment.

2.5. Cell diameter determination

To measure cell diameter of *Chlorella* sp., 0.5 mL of culture were obtained at the end of the experiment and examined under a microscope at 400× magnification. The average diameter of 30 randomly selected cells was measured using an ocular micrometer.

2.6. Chlorophyll-*a* determination

To quantify the Chl-*a*, a known volume of the culture solution (10 mL) was filtered through Whatman GF/C filter paper at the end of the experiment. Subsequently, the Chl-*a* content was extracted using 90 % acetone and incubated overnight in darkness at 4 °C. Following centrifugation, the supernatant was collected and used to measure the concentration of Chl-*a* at wavelengths ranging from 630 to 750 nm using a UV-VIS spectrophotometer (Harch, model 500). The Chl-*a* concentration ($\mu\text{g/mL}$) was determined and calculated following the method outlined by APHA (2005).

2.7. Data analyses

The half maximal effective concentration (EC_{50}) values for SMX on *Chlorella* sp. at 24 h and 96 h were determined through linear regression, correlating the logarithm of SMX concentration (x) with microalga growth inhibition (y). All data in this study are presented as mean \pm SD. To assess statistical differences between SMX and control treatments, a one-way analysis of variance (ANOVA) was conducted, followed by post-hoc Tukey's honestly significant difference (HSD) test. Prior to analysis, log transformation was applied to ensure a normalized distribution of all data. Significance was considered at P-values < 0.05 .

3. RESULTS AND DISCUSSION

3.1. Acute toxic effects

In comparison to the control group, exposure of SMX to *Chlorella* sp. induces acute toxic effects on the growth of the microalgae. The $EC_{50-24\text{h}}$ and $EC_{50-96\text{h}}$ of SMX on the growth inhibition of *Chlorella* sp. were 0.56 mg/L and 0.41 mg/L, respectively.

In previous studies, the acute toxic effects of SMX on several aquatic organisms including microalgae, crustacean, amphibian and zebrafish have been investigated (Borecka et al., 2016; Liu et al., 2020; Xu et al., 2022; Zhang et al., 2023). In microalgae, Xiong et al. (2019) and Zhang et al. (2021) reported the $EC_{50-96\text{h}}$ values of 0.12 mg/L and 0.49 mg/L for SMX against the green microalgae *Scenedesmus obliquus* (*S. obliquus*) and *R. subcapitata*, respectively. In the current investigation, the $EC_{50-96\text{h}}$ of SMX against the green microalga

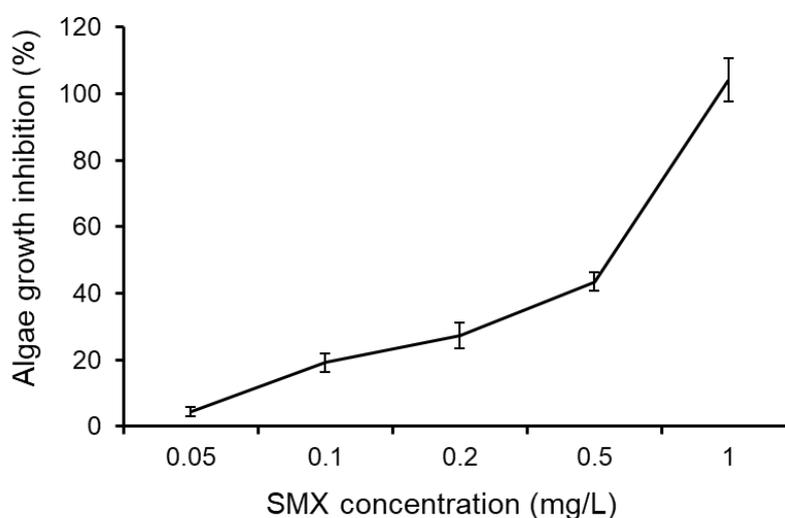


Figure 2. The effects of SMX on the growth inhibition of *Chlorella* sp.

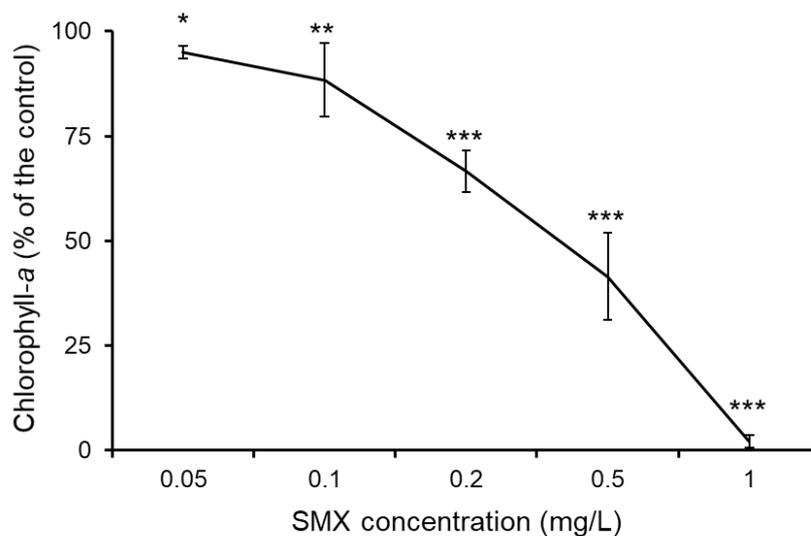


Figure 3. The average Chl-*a* concentrations. Asterisks denote the degree of differences between treatments and the control group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Chlorella sp. is determined to be 0.41 mg/L. This value is slightly higher than the EC_{50-96} h for *S. obliquus* but slightly lower than that for *R. subcapitata*. These findings indicate that the tropical freshwater *Chlorella* sp. utilized in this study may exhibit higher sensitivity to SMX compared to *R. subcapitata* but lower sensitivity than *S. obliquus*. Future studies need to be conducted to confirm the sensitivity of various microalgae species originating from temperate, sub-tropical, and tropical environments to SMX and other antibiotics.

3.2. Sub-chronic toxic effects

3.2.1 Algae growth inhibition

The sub-chronic toxic effects of SMX on the growth inhibition of *Chlorella* sp. was showed in Figure 2. The results revealed a pronounced inhibitory effect of SMX on algal growth, with significant and dose-dependent increases in growth inhibition observed. Statistically significant differences were detected at all concentrations of SMX tested. Specifically, SMX concentrations of 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, and 1.0 mg/L resulted in inhibitions of 4.4 %, 19.2 %, 27.4 %, 43.5 %, and 104.2 % of *Chlorella* sp., respectively. Results showed that the concentration of 1 mg/L, SMX completely inhibited the growth of *Chlorella* sp. (Figure 2).

Previous studies have reported that antibiotics can adversely affect the growth and reproduction of various microalgae. These effects may result from influences on algal cell structure, reduction in chlorophyll content, induction of oxidative stress, or the generation of reactive oxygen species (Borecka et al., 2016; Xiong et al., 2019; Zhang et al., 2021; Xu et al., 2022). The mechanism by which SMX inhibits

microalgae involves disrupting folic acid metabolism and the permeability of glutamate channels on cell walls, which are precursors for protein synthesis crucial to algae growth and reproduction (Xu et al., 2022). Additionally, SMX exhibits various toxic effects, encompassing cytotoxic, mutagenic, and carcinogenic properties (Zhang et al., 2021). Antibiotics can lead to swift mortality in certain microalgae, causing a decrease in cell density within the exposure treatments (Xiong et al., 2019; Xu et al., 2022). Our findings closely align with prior research, indicating that SMX demonstrates diverse toxic effects on the growth and reproduction of microalgae.

3.2.2. Chlorophyll-*a* concentration

Figure 3 shows the average Chl-*a* concentrations of *Chlorella* sp. treatments following exposure to varying concentrations of SMX, in comparison to the control. The results indicated that SMX triggers a decline in Chl-*a* concentration across all treatments, with significant and dose-dependent increases in the reduction of Chl-*a* content observed. Specifically, SMX concentrations of 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, and 1.0 mg/L resulted in decreases of 5 %, 12 %, 33 %, 49 %, and 98 % in Chl-*a* content, respectively.

Chlorophylls serve as the predominant natural pigments essential for oxygenic photosynthesis in microalgae, harnessing solar energy for metabolism and reproduction (Khoo et al., 2023). In green microalgae, specifically Chlorophyceae, prominently feature Chl-*a* as their main pigment, imparting a green color. In eukaryotic green microalgae, chlorophylls are situated within the chloroplast (da Silva & Lombardi, 2020).

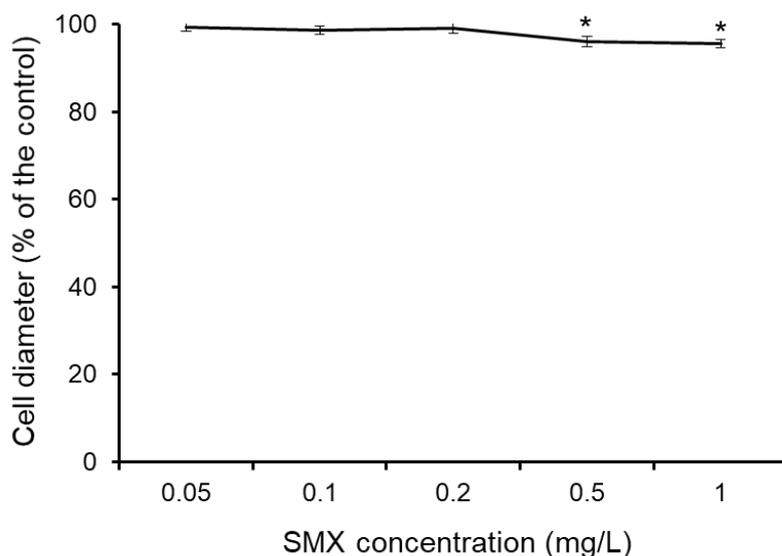


Figure 4. The average cell diameter of *Chlorella* sp. Asterisks denotes the degree of differences between treatments and the control group (* $p < 0.05$).

Typically, antibiotics disrupt both cell wall and chloroplast structures in microalgae, leading to heightened membrane permeability and reduced chlorophyll content (Borecka et al., 2016; Xiong et al., 2019). Antibiotics can exert an influence on the photosynthetic process of microalgae by suppressing the functionality of chloroplast ribosomes as well (Xu et al., 2022). Our findings align with earlier observations indicating a substantial decrease in Chl-*a* levels in green microalgae exposed to SMX (Zhang et al., 2021; Xu et al., 2022). In addition to these effects on chloroplasts, exposing microgreen algae to antibiotics has been observed to increase mitochondrial numbers and volume within algal cells, inducing stress conditions or the generation of ROS. Specifically, SMX has been noted to damage chloroplasts and mitochondria, triggering substantial ROS production in microgreen algae, such as *S. obliquus* (Xu et al., 2022).

3.2.3. Cell diameter

Figure 4 shows the average cell diameters of the green algal *Chlorella* sp. following a 10-day exposure to various concentrations of SMX. In the control group, the recorded average cell diameter ranged from $7.9 \pm 0.8 \mu\text{m}$. There was no significant difference in the cell diameter of *Chlorella* sp. observed when exposed to SMX at concentrations of 0.05 mg/L, 0.1 mg/L, and 0.2 mg/L. Nonetheless, notable differences in algal cell diameters were observed when exposed to SMX at concentrations of 0.5 mg/L and 1.0 mg/L, resulting in a reduction ranging from 4.0 % to 4.5 % in cell diameter.

Previous studies have primarily focused on the acute toxic effects of SMX on microalgae,

specifically examining biological responses or toxicity at the molecular level (Borecka et al., 2016; Xiong et al., 2019; Zhang et al., 2021; Xu et al., 2022), however, little is known about the sub-chronic toxic effects on cell morphology. The impact of antibiotics on the shape, size, intracellular structures, and cell wall of microalgae remains unclear. Our study revealed that SMX leads to a reduction in the cell diameter of *Chlorella* sp. Xu et al. (2022) documented that exposure to SMX induced gene expression in the microgreen algae *Scenedesmus*, leading to alterations in algal cell ultrastructure.

Xu et al. (2022) highlighted the toxicity mechanisms of SMX on the microgreen algae *Scenedesmus*, involving alterations such as blurring in the algal cell wall, damage to chloroplast structure, and variations in the number and volume of mitochondria within the cell. These changes subsequently led to a reduction in chlorophyll pigments, induced cell wall permeability, and inhibited cell growth and reproduction. It is likely that SMX induces various adverse effects on the intracellular structure and cell wall of microgreen algae, consequently resulting in a decrease in the cell diameter of the tested algae. Further research is necessary to comprehensively understand the specific disruptions caused by antibiotics and SMX on the shape, size, intracellular structures, and cell wall of various microalgae.

4. CONCLUSIONS

This study examined the toxic effects of SMX at environmentally relevant concentrations on the tropical freshwater microalga *Chlorella* sp. cultivated

under laboratory conditions. The results indicated that SMX induced both acute and sub-chronic toxic effects on the tested algae. The EC₅₀-24h and EC₅₀-96h values for SMX on the freshwater microalga *Chlorella* sp. were estimated to be 0.56 mg/L and 0.41 mg/L, respectively. Sub-chronic exposure to SMX exhibited a pronounced inhibitory effect on algal growth, showing significance and dose-dependency. Additionally, all SMX treatments demonstrated impairment in chlorophyll. Higher concentrations of SMX also led to a reduction in the cell diameter of *Chlorella* sp. This study contributes valuable information on the acute and sub-chronic toxicity of environmentally relevant concentrations of antibiotics on tropical freshwater microalgae.

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