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Biochemical responses of the freshwater microalga *Dictyosphaerium* sp. upon exposure to three sulfonamides

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ABSTRACT

Sulfonamides (SAs) are common antimicrobial drugs, which are frequently detected in surface water systems, and are difficult to degrade, posing a potential threat to the aquatic environment. However, little is known about the potential adverse effects of SAs on non-target organisms (e.g., microalgae) in the aquatic ecosystem. In this study, the effect of SAs (sulfadiazine (SD), sulfamerazine (SM1), and sulfamethazine (SM2) at 1, 5, 20, and 50 mg/L concentrations, respectively) on the freshwater microalga *Dictyosphaerium* sp. was investigated, with respect to changes of biomass and chlorophyll *a* content and induction of extracellular polymer substances (EPS), including protein and polysaccharide contents. At the same time, the residue of SAs was determined. The results showed that *Dictyosphaerium* sp. was tolerant to the three SAs, and the chlorophyll *a* content in *Dictyosphaerium* sp. significantly decreased on day 7, followed by a "compensation phenomena". The increase in protein and polysaccharide contents played a defensive role in *Dictyosphaerium* sp. against antibiotic stress, and there was a strong positive correlation between polysaccharide contents and antibiotic concentrations. *Dictyosphaerium* sp. exhibited 35%–45%, 30%–42%, and 26%–51% removal of SD, SM1, and SM2, respectively. This study is helpful to understand the changes of EPS in the defense process of microalgae under the action of antibiotics, and provides a new insight for the ecological removal of antibiotic pollution in natural surface water system.

Introduction

The global demand for antibiotics for use in humans, animal husbandry, and aquaculture applications has been constantly increasing (Wang et al., 2017), and 17%–90% of antibiotics cul-

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minate in the environment through urine and feces from humans and animals (Kiki et al., 2020). Sulfonamides (SAs) are the first antibiotics to be systematically used to protect humans and animals from infectious diseases (Xiong et al., 2019a). Sulfadiazine (SD), sulfamerazine (SM1), and sulfamethazine (SM2) contain a pyrimidine ring, which are widely found in surface water, groundwater, soil, and even drinking water, with concentrations up to μg/L level (Chen et al., 2019). While the toxicity of antibiotics against pathogenic bacteria is well known, there is a paucity of data about their ecotoxicity to non-target organisms (Migliore et al., 1997), especially microalgae.

Microalgae are abundant in aquatic habitats and play a key role in the entire biosphere (Yang et al., 2002). They serve as primary producers representing the foundation of the aquatic food web and produce 70% of the Earth's oxygen content (Reynolds, 1984). However, perturbations and alterations of their primary production may severely affect other biotic communities (Bartolomé et al., 2009). Hence, any adverse effects of xenobiotics on phytoplanktonic populations may have serious ecological consequences. When dealing with xenobiotics, the mechanisms by which microalgae produce enzymatic and non-enzymatic antioxidants to protect themselves are well known. For example, superoxide dismutase (SOD) activity and malondialdehyde (MDA) level in *Chlamydomonas mexicana* were reported to markedly increase in the presence of ciprofloxacin (Xiong et al., 2017). Wan et al. (2015) demonstrated that the activities of SOD and catalase (CAT), and the level of MDA in erythromycin-treated *Microcystis flos-aquae* were stimulated.

Extracellular polymer substances (EPS) are composed of secretions from microorganisms and cellular lysis products (Dong et al., 2017), and a wide range of bacteria synthesize and secrete EPS that are involved in bacterial tolerance to environmental stresses (Han et al., 2017; Pereira et al., 2009). They consist primarily of proteins and polysaccharides, with nucleic acids and lipids as minor constituents (Wang et al., 2019). Proteins and polysaccharides also are the main organic groups in microalgal biomass (Markou et al., 2012). However, few reports have indicated that the contents of proteins in microalgae under pesticide (cypermethrin) and surfactant (nonylphenol) stress were increased (Cheng et al., 2020; Gao et al., 2016), and that the presence of silver nanoparticles and ions and quaternary ammonium compounds promoted the microalgae to secrete more EPS (Li et al., 2019; Zheng et al., 2019). In a word, the composition of EPS response to antibiotic stress in green algae has rarely been evaluated.

In this study, to investigate the response of microalgae to SAs-induced oxidative stress, especially with respect to protein and polysaccharide contents, *Dictyosphaerium* sp. was selected as an optimal microalgal species owing to its high EPS content and high flocculation activity (Halaj et al., 2018; Wang et al., 2019). The toxicity of different concentrations of SD, SM1, and SM2 to *Dictyosphaerium* sp. was evaluated based on algal growth, chlorophyll *a* content, total protein content, and polysaccharide [including water-soluble released polysaccharides (RPS) and capsular polysaccharide (CPS)] content. In addition, the removal of the three SAs by the alga was also analyzed.

1. Materials and methods

1.1. Chemicals

SD (CAS-No. 68-35-9), SM1 (CAS-No. 127-79-7), and SM2 (CAS-No. 257-68-1) with > 98% purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the three test antibiotics were prepared using BG-11 medium. We used 0.1 mol/L NaOH as solvent to maintain pH 10 to fully dissolve SAs. Then, we added 0.1 mol/L HCl to adjust the initial pH 7 of the test medium (Chen et al., 2020). Methanol and water (LCMS grade) were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

1.2. Tested organisms

Axenic strain of *Dictyosphaerium* sp. (Code: FACHB-1902), originally purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-Collection), Wuhan City, China, was cultivated in 3000-mL Erlenmeyer flasks containing BG-11 medium (Rippka et al., 1979). All experimental devices used for algal culture were autoclaved at 121°C for 30 min before use. The algal cultures were maintained in a homoeothermic incubator at 25 ± 1°C under 4000 lx illumination with a light-dark period of 12:12 hr. To reduce any effect caused by minor differences in photon irradiance, the flasks were arranged randomly and gently shaken three times a day (Wan et al., 2015).

1.3. Experimental design

Tests were performed according to guideline No. 201 of the Organization for Economic Cooperation and Development (OECD) with minor modifications (OECD, 2011). The experiment was conducted in 250-mL Erlenmeyer flasks containing 150 mL tested solution of algae and antibiotics for 16 days under the same conditions used for the inoculum culture. There are two main steps: a specific volume of the algal culture was diluted with BG-11 medium to achieve a constant cell fresh weight (80 mg/L). Then, each antibiotic stock solution was added to the algal medium to obtain final antibiotic concentrations of 1, 5, 20, and 50 mg/L, respectively. The control comprised algal medium without added antibiotics. Samples were collected on days 1, 2, 4, 7, 11, and 16, unless otherwise stated. Each test concentration was prepared in triplicate, and all the experiments were performed under sterile conditions to avoid bacterial contamination.

1.4. Measurement of cell growth and fluorescent chlorophyll a content

The algal cell growth was determined by analyzing the optical density (OD) of the samples (3 mL) from each treatment at a maximum absorption wavelength of 688 nm using an ultraviolet-visible spectrophotometer (Unico UV-2800, Shanghai, China). The value obtained was converted to algal wet biomass based on the linear relationship between OD_{688} and cell biomass (mg/L) as follows:

$$\text{Cell biomass} = 749.97 \times OD_{688} - 4.6434 \left(R^2 = 0.999\right) \quad (1)$$

The specific growth rate (μ) was calculated by fitting the algal biomass to an exponential function using the following Eq. (2):

$$\mu = (\ln N_2 - \ln N_0)/(t_2 - t_0) \quad (2)$$

where N_2 (mg/L) is the algal biomass at time t_2 (day) and N_0 (mg/L) is the algal biomass at time t_0 (day).

The chlorophyll *a* fluorescence content of the alga was measured using a pulse amplitude-modulated fluorometer (Phyto-PAM, Walz, Effeltrich, Germany) equipped with an emitter-detector-fiberoptic unit with an irradiance of 16 μmol photons/m^2/sec PAR.

1.5. Cell harvesting and disruption

During exposure to antibiotic solutions, changes in algal total protein and polysaccharide (RPS and CPS) contents were determined. The algal cell suspension (5 mL) was harvested by centrifugation at 6000 × *g* for 15 min (Eppendorf, Centrifuge 5804R). The supernatant (0.5 mL) was diluted with deionized water to 2 mL to measure the RPS. The harvested cells were re-suspended in 0.4 mL of phosphate buffer solution (PBS) and homogenized using a manual homogeniser for 10 min. The homogenate was diluted with PBS to 2 mL and then centrifuged at 6000 × *g* for 5 min. The supernatant obtained was used for subsequent assays.

1.6. Measurement of total protein and polysaccharide contents

The algal total protein content was determined using Bradford's method (Bradford, 1976) with bovine serum albumin as standard and expressed as milligram per milligram algal fresh weight (FW). In brief, 0.5 mL of the collected supernatant was mixed with 5 mL of Coomassie Brilliant Blue (G-250), and after 30 min, the absorbance was recorded at 595 nm. The RPS and CPS contents were determined using the anthrone-ethyl acetate–sulfuric acid method (Wang, 2006) using glucose as standard. In brief, 0.5 mL of the collected supernatant was diluted with PBS to 2 mL as the CPS tested solution, and the 2 mL diluted CPS and RPS supernatants were mixed with 0.5 mL of anthrone-ethyl acetate and 5 mL of 98% sulfuric acid, respectively, followed by a vigorous shaking. Subsequently, the colorimetric tube was placed in a hot bath for 10 min, and after cooling, the absorbance was recorded at 620 nm. The sugar content (%) was calculated from a standard curve of glucose as follows:

$$\text{sugar content} = \left[(m \times V_T \times N)/\left(m_S \times V_S \times 10^3\right)\right] \times 100\% \quad (3)$$

where m (μg) is the glucose content determined by standard linear equations, V_T (mL) is the extract volume, N is the diluted multiples, m_S (mg) is the sample weight, and V_S (mL) is the measured volume.

1.7. Determination of antibiotic concentration

The concentrations of antibiotics in the algal cultures were measured using ultra performance liquid chromatography mass spectrometry (UPLC–MS/MS). The algal culture samples were filtered through 0.22-μm polytetrafluoroethylene filters prior to use for UPLC–MS/MS analysis. The LC column was a CORTES UPLC C_{18} chromatography column with particle size of 1.6 μm and dimension of 100 × 2.1 mm employed at a constant flow rate of 0.3 mL/min. A total of 2 μL of each sample were injected using an auto-sampler. The mobile phase consisted of a mixture of solution A (0.1% formic acid in LCMS-grade water) and solution B (LCMS-grade methanol) with an initial composition of 10% B. The mobile phase composition was changed from 10% B at 1 min to 98% B at 4 min, after which the column was rinsed with 98% B for 2 min and re-equilibrated to initial conditions for another 2 min. The total running time was 8 min. All the experiments were performed using a Waters Xevo TQ-S triple-quadrupole mass spectrometry (MS), in which the MS transition (precursor ion → product ion) was 251.1 → 108.3 for SD, 265.1 → 156.1 for SM1, and 279.4 → 186.2 for SM2. All the analytes were measured in electrospray positive (ESI^+) mode.

The residual antibiotic (%) of SAs was calculated using the following Eq. (4):

$$\text{residual antibiotic} = C_t/C_0 \times 100\% \quad (4)$$

where C_0 (mg/L) is the initial concentration of antibiotics at time 0 and C_t (mg/L) is the concentration of antibiotics at time t.

1.8. Statistical analysis

One-way analysis of variance, followed by least significant difference post hoc test, was employed to identify the significant differences between the control and treatment groups using the statistics software SPSS 17.0 (Chicago, IL, USA). Prior to the analysis, normality of the data was evaluated by using Shapiro-Wilk test, and homogeneity of variances was checked by Levene's test. The differences were considered to be significant and very significant at $p < 0.05$ and $p < 0.01$, respectively. All figures were generated using Origin 2017 software (OriginLab, Northampton, MA, USA). Spearman's correlation coefficient was used to compare the strength of the relationship between antioxidant contents and antibiotic concentrations.

2. Results

2.1. Degradation of SD, SM1, and SM2

The removal of SD, SM1, and SM2 by *Dictyosphaerium* sp. was investigated during the algal exposure period (Fig. 1). In all the treatment groups with 5 mg/L SAs, the residual antibiotics reached the minimum at the earliest, namely, on days 2, 4, and 4, respectively. The maximum removal rates of the three SAs in all the treatment groups by *Dictyosphaerium* sp. were from 35% (50 mg/L) to 45% (5 mg/L) for SD, from 30% (50 mg/L) to 42% (1 mg/L) for SM1, and from 26% (50 mg/L) to 51% (1 mg/L) for SM2 after 16 days of exposure.

2.2. Effect on algal growth

Different concentrations (1, 5, 20, and 50 mg/L) of the three SAs showed almost consistent effect on the growth of *Dictyosphaerium* sp. (Fig. 2), and no growth inhibition was noted upon antibiotic exposure. The algal biomass significantly increased in some treatment groups; for example, when compared with the control, algal biomass increase was detected in the 1 mg/L SD treatment group on day 2, 1 and 5 mg/L SM1 treatment groups on day 2, 5 and 20 mg/L SM2 treatment groups on day 1, and 1 and 5 mg/L SM2 treatment groups on day 2. However, on day 16, only the 1 mg/L SD and 5 mg/L SM1 and SM2 treatment groups presented higher biomass, when compared with that of the control.

The μ of *Dictyosphaerium* sp. increased with the increasing experiment period up to day 4, and then declined (Appendix A **Fig. S1**). In particular, on day 1, *Dictyosphaerium* sp. treated with 50 mg/L SD, 5 and 20 mg/L SM1, and 1 and 5 mg/L SM2 showed a relatively low μ, when compared with that of the control. However, after 7 days, there were only slight variations among the treatment groups, when compared with the control. The μ in some treatments that initially was strongly suppressed by the SAs was higher than that of the control at the end of the exposure period (i.e., 50 mg/L SD and 5 mg/L SM1).

2.3. Effect on biochemical assays

The chlorophyll *a* content of *Dictyosphaerium* sp. exposed to four concentrations of the three SAs is illustrated in Fig. 3. SD, SM1, and SM2 produced a significant decrease in the algal content on days 4, 2, and 1, respectively. On day 7, all the treatment groups showed significant decrease in the algal chlorophyll *a* content. However, the content of chlorophyll *a* was close to that of the control group at day 11. On day 16, the chlorophyll *a*

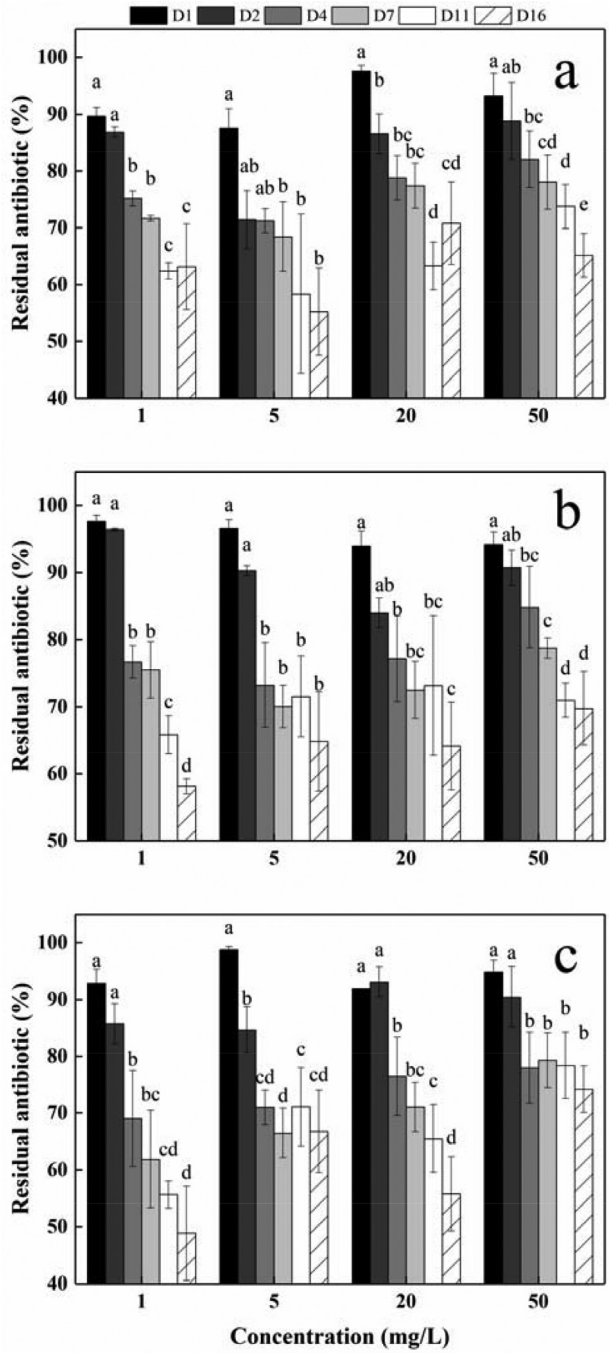

Fig. 1 – Residual antibiotic (%) of different concentrations of (a) SD, (b) SM1, and (c) SM2 by *Dictyosphaerium* sp. during 16 days of exposure. Error bars represent the standard error of the mean (*n*=3). Columns with different letters indicate significant differences ($p < 0.05$) between the control and treatment groups.

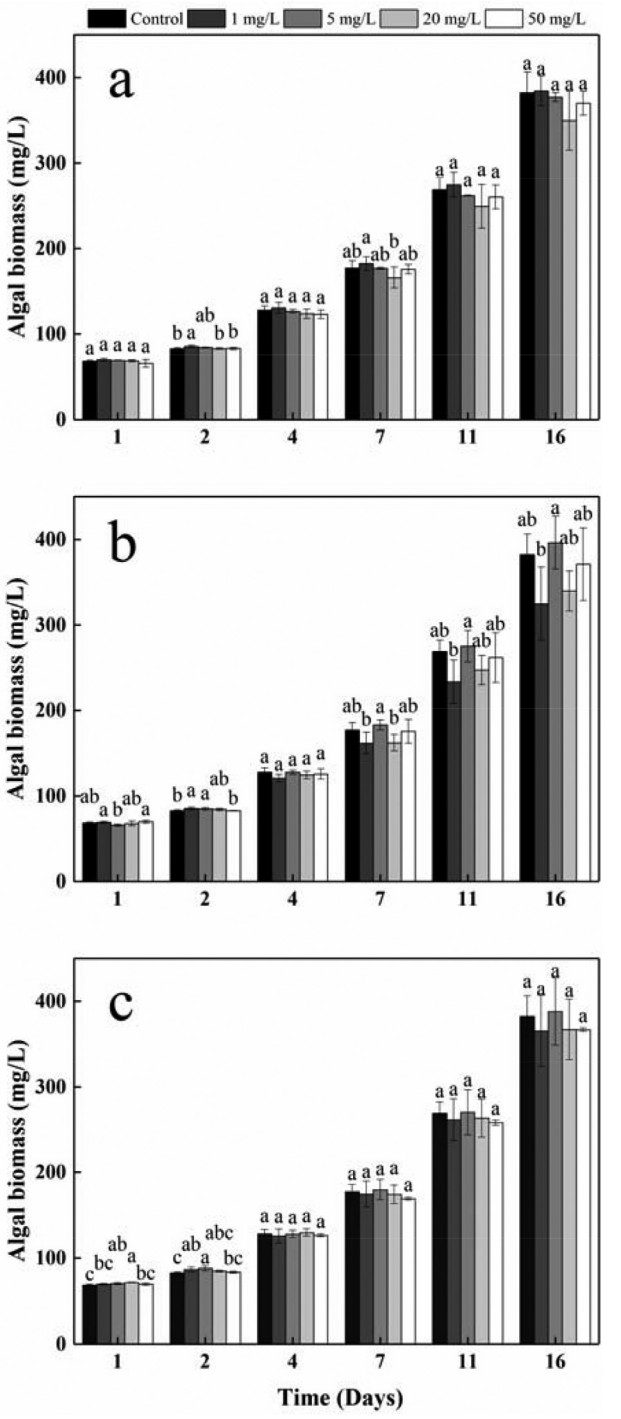

Fig. 2 – Effect of different concentrations of (a) SD, (b) SM1, and (c) SM2 on the biomass of *Dictyosphaerium* sp. during 16 days of exposure. Error bars represent the standard error of the mean (*n*=3). Columns with different letters indicate significant differences ($p < 0.05$) between the control and treatment groups.

content in all the treatment groups was lower than that in the control, and maximum inhibition ratios of 16.1%, 12.2%, and 17.7% were noted in groups treated with 20 mg/L SD, 20 mg/L SM1, and 50 mg/L SM2, respectively.

The total protein content in *Dictyosphaerium* sp. during the 16-day exposure period was analyzed. In the control, the algal protein content was almost constant. However, in

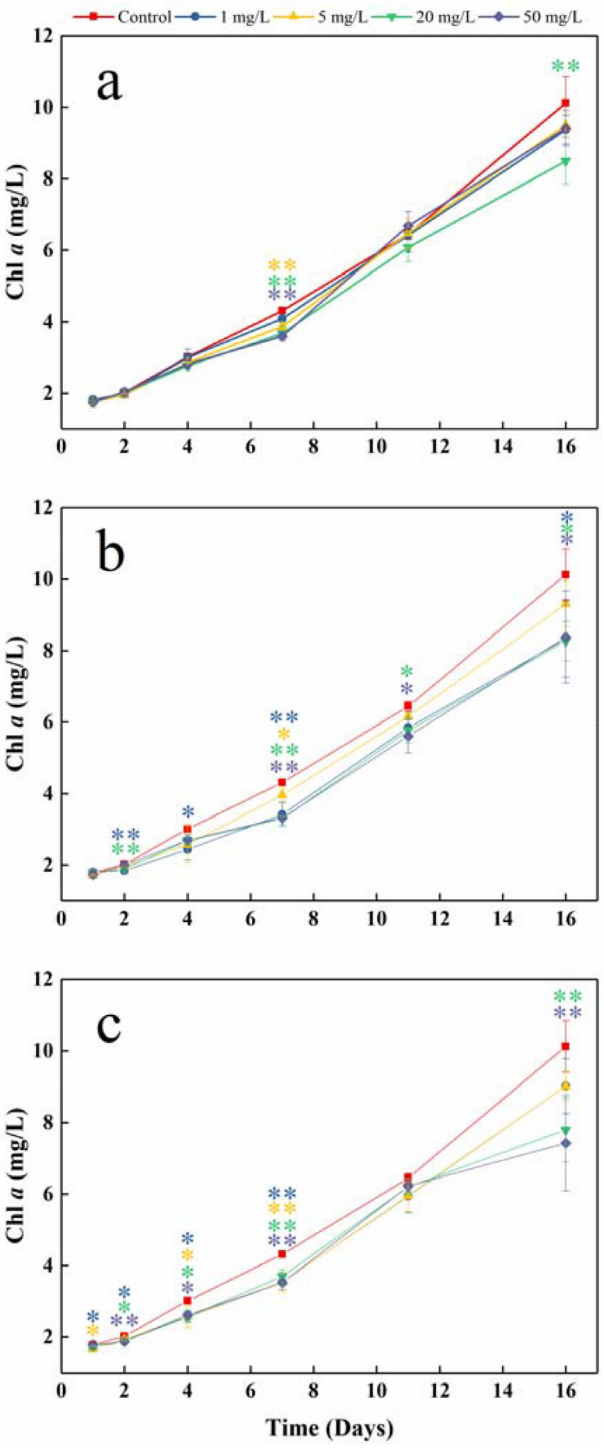

Fig. 3 – Effect of different concentrations of (a) SD, (b) SM1, and (c) SM2 on the chlorophyll *a* content of *Dictyosphaerium* sp. during 16 days of exposure. Error bars represent the standard error of the mean (*n*=3). Asterisks indicate significant differences ($p < 0.05$) between the control and treatment groups. *$p < 0.05$; **$p < 0.01$.

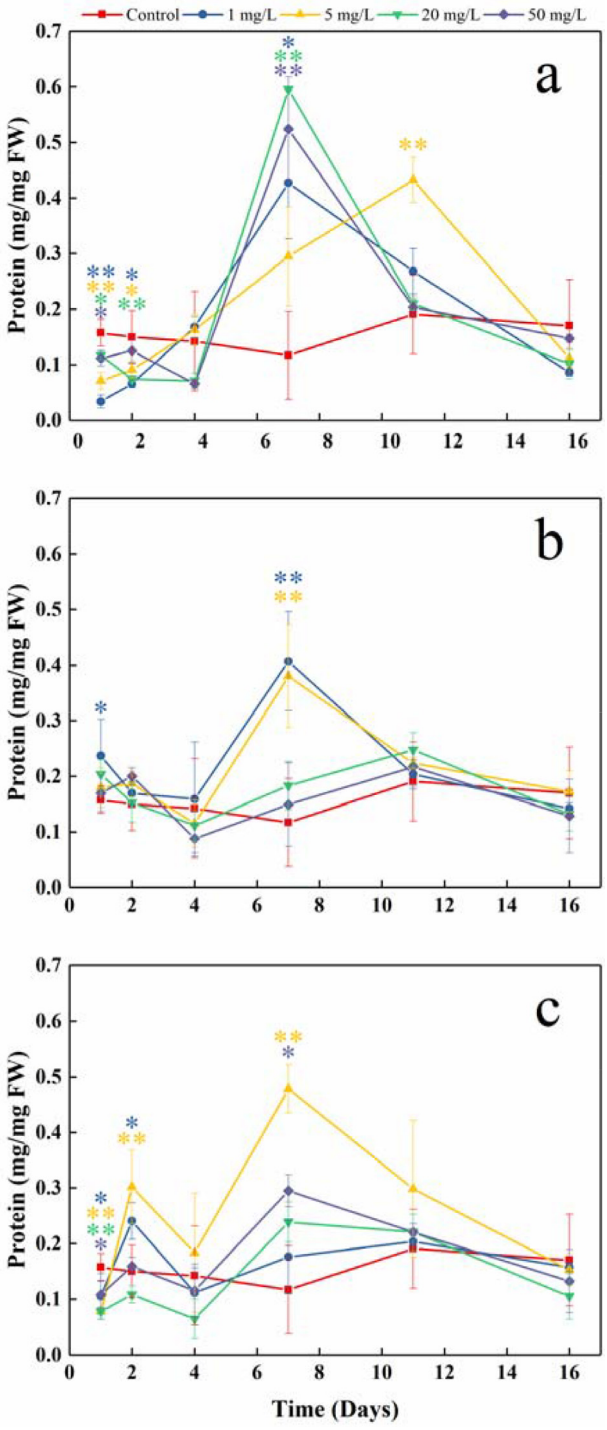

Fig. 4 – Effect of different concentrations of (a) SD, (b) SM1, and (c) SM2 on the total protein content of *Dictyosphaerium* sp. during 16 days of exposure. Error bars represent the standard error of the mean (*n*=3). Asterisks indicate significant differences ($p < 0.05$) between the control and treatment groups. *$p < 0.05$; **$p < 0.01$.

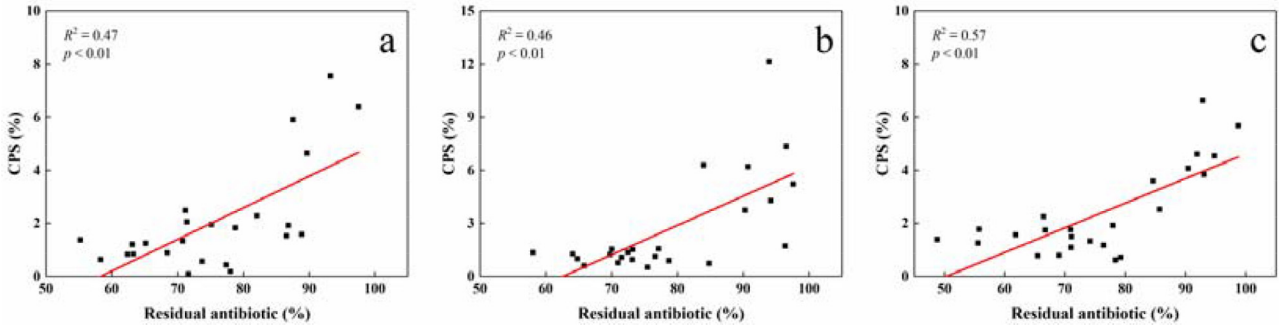

Fig. 5 – Relationship between CPS and (a) SD, (b) SM1, and (c) SM2.

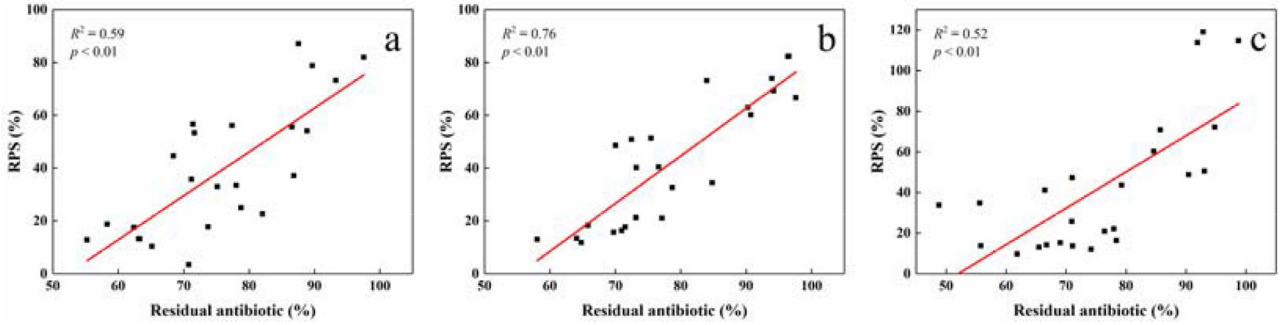

Fig. 6 – Relationship between RPS and (a) SD, (b) SM1, and (c) SM2.

the SD and SM2 treatment groups, the algal protein content significantly decreased, when compared with the control, on day 1 (Fig. 4). On day 7, almost all of treatment groups presented marked increases in the protein contents, which in SD-, SM1-, and SM2-treatments were about 5.09-fold, 3.48-fold, and 4.09-fold higher than that of the control, respectively. However, on day 16, the algal protein content in the treatment groups was lower than that in the control group.

The changes in the algal CPS and RPS contents during the 16-day exposure are shown in Appendix A Fig. S2 and Fig. S3, respectively. The CPS content in the control group was relatively high in the initial 2 days and gradually decreased over the next 14 days. On day 1, the CPS content in *Dictyosphaerium* sp. treated with 50 mg/L SD and 5 and 20 mg/L SM1 was significantly higher than that in the control. Subsequently, the CPS content in all the treatment groups dramatically decreased until day 7, especially on day 2, the CPS content in SD-, SM1-, and SM2-treatments were about 4.23-fold, 3.76-fold, and 2.55-fold lower than that of the control, respectively. After day 7, the CPS content in all the treatment groups was slightly higher than that in the control. The variations in the RPS content in the control were similar to those in the CPS content, but the RPS level was lower on day 2. The RPS content in all the treatment groups was consistently higher than that in the control during the 16-day exposure period, and presented a significant decrease until day 4, followed by another peak on day 7, when the RPS content in SD-, SM1-, and SM2-treatments were about 5.75-fold, 5.26-fold, and 4.83-fold higher than that of the control, respectively. In the present study, a linear regression analysis between CPS and RPS contents and residual antibiotics was conducted. A strong positive correlation was observed between the algal CPS and RPS contents and antibiotic concentrations (Figs. 5 and 6), respectively.

3. Discussion

Cell biomass and μ were commonly used to quantify the growth of microalgae (Qian et al., 2018). An increase in algal biomass has also been reported for *Scenedesmus obliquus* treated with a mixture of SM2 and sulfamethoxazole (Xiong et al., 2019b), *Tetraselmis chuii* treated with oxytetracycline and florfenicol (Ferreira et al., 2007), and *Chlorella pyrenoidosa* and *Isochrysis galbana* treated with chloramphenicol and *I. galbana* treated with florfenicol (Lai et al., 2009), which may be owing to the consumption of organic compounds as nutritional sources (Kiki et al., 2020). The algal biomass at high concentration of SAs was lower than that of the control group after long-term exposure, indicating that antibiotics had a chronic toxic effect on algal cells (Kumar et al., 2015). A "compensation phenomena" occurred in algal cells, that is, the relatively low μ value at the early stage of the exposure and the relatively high μ value at the end of the exposure, possibly because it absorbed nutrients at a slower rate, when compared with that in the other treatment groups. These results indicated that SAs had low toxicity to *Dictyosphaerium* sp.; in other words, the alga was highly tolerant to these antibiotics. According to the results of changes in μ values, the acute toxicity of the three SAs showed the following trend: SM2 > SM1 > SD.

The photosynthetic pigment content is a sensitive parameter under environmental stress conditions (Wang et al., 2017). In general, the chlorophyll *a* content of *Dictyosphaerium* sp. exhibited distinct changes under different SAs treatments. The response time of chlorophyll *a* to the toxic effects of the three SAs was significantly different, indicating that *Dictyosphaerium* sp. was highly susceptible to SM2, followed by SM1, and then SD. The possible reasons for the decrease in the algal chlorophyll *a* content is related to the disintegration of thylakoid or cell membrane under antibiotic stress (Cai et al., 2009;

Wan et al., 2014) and the accumulation of intracellular ROS in the algal cells (Eguchi et al., 2004; Isidori et al., 2005), which cannot prevent the interaction of toxic chemicals with intracellular organelles, resulting in the disruption of microalgal chlorophyll synthesis. However, on day 11, even though the alga was knocked down under initial exposure conditions and the "compensation phenomena" occurred, this may be due to the decrease in the antibiotic concentrations with time or the nontoxic effect of antibiotics on the algal biomass after 7 days of exposure. Similar observations have been reported for other algae such as *M. flos-aquae* and *Chlorella vulgaris* (Kurade et al., 2016; Wang et al., 2017). Kurade et al. (2016) proved that the algal chlorophyll *a* content had a significant positive correlation with algal biomass, while Wang et al. (2017) mentioned that the increase in chlorophyll *a* content can enhance algal photosynthesis, which can stimulate higher energy accumulation in the algae and promote algal growth and reproduction.

Proteins and polysaccharides are important components in EPS due to their special functions and characteristics (Wang et al., 2019). Changes in the total protein content may be owing to the toxicity of the SAs leading to ROS generation (Leitão et al., 2003) and the response of algal cells to oxidative stress. Xiong et al. (2019b) reported that the increase in the protein content in microalgae under environmental stress may indicate the synthesis of defensive proteins (e.g., stress-responsive proteins). Similarly, Gao et al. (2016) also confirmed that protein contents in *C. vulgaris* were significantly increased after exposure to the pesticide, cypermethrin. At the end of the exposure, the lower protein content of all SAs treatment groups than the control group may be attributed to SAs inhibition of cell proliferation or nutrient deficiency.

The increase in CPS may be owing to the diversion of energy production towards synthesis of storage compounds (polysaccharides) under antibiotic stress (Kumar et al., 2015) and the recovery of chlorophyll synthesis activity in the algal cells (Kumar et al., 2015). This increase in RPS accumulation may be an adaptive response of the alga against free antibiotics (Wang et al., 2014), and antibiotics may affect the carbon cycle, causing cells to secrete large amounts of RPS to achieve carbon balance (Ren et al., 2013). To the best of our knowledge, this study is the first to report on the changes in the CPS and RPS contents of green algae under antibiotic stress. As CPS has been reported to play a significant role in heavy metal removal by cyanobacteria (Weckesser et al., 1988), a correlation analysis between CPS and RPS contents and antibiotic concentrations was done in the present study, which presented a significant correlation, i.e., the polysaccharide contents increased with the increase in the antibiotic concentrations. These results achieved in the present study could be attributed to the role of large amounts of EPS in *Dictyosphaerium* sp., especially the high polysaccharide content (Halaj et al., 2018), which is conducive to the removal of antibiotics through adsorption, accumulation, or degradation of *Dictyosphaerium* sp. (Cheng et al., 2020), that is, the removal rate of SAs by *Dictyosphaerium* sp. was higher than that of other green algal species, such as *S. obliquus* (Xiong et al., 2019a), *Nannochloris* sp. (Bai and Acharya, 2016), and *C. vulgaris* (Zhang and Ma, 2013) in previous studies, whose removal rate of SAs was only 17%–32%.

4. Conclusions

SD, SM1, and SM2 (at up to 50 mg/L concentration) had no significant negative effect on the growth of *Dictyosphaerium* sp. However, alteration in the chlorophyll *a* content indicated that the SAs caused photosynthesis changes in *Dictyosphaerium* sp. The total protein and polysaccharide contents were elevated in the alga, and a strong positive correlation between polysaccharide contents and antibiotic concentrations was detected, which indicated the defensive response of *Dictyosphaerium* sp. against the three SAs. In the future, the roles of polysaccharides in algae under antibiotic stress must be clarified through biochemical and structural analyses, which can provide a basis for understanding the mechanism of EPS produced by algae to remove antibiotics.

Acknowledgments

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.05.018.

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