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Growth response and toxic effects of three antibiotics on *Selenastrum capricornutum* evaluated by photosynthetic rate and chlorophyll biosynthesis

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Abstract

The effects of three types of antibiotics (erythromycin, ciprofloxacin and sulfamethoxazole) on the photosynthesis of freshwater algae, *Selenastrum capricornutum* Printz, were investigated by determining the growth rate, chloroplast pigments content, seven main precursors (including δ-aminolevulinic acid, porphobilinogen, uroporphyrinogen III, coproporphyrinogen III, protoporphyrin IX, Mg-proporphyrin IX and protochlorophyllide), and photosynthetic rate during chlorophyll biosynthesis. The antibiotics significantly decreased the growth rate, chlorophyll content, and photosynthetic rate. Erythromycin induced a decreasing effect at a concentration of 0.06 mg/L, while ciprofloxacin and sulfamethoxazole achieved the same results at concentrations higher than 1.5 mg/L. Only erythromycin significantly inhibited chlorophyll biosynthesis, which indicated that it was considerably more toxic to *S. capricornutum* than ciprofloxacin and sulfamethoxazole, and may pose a high potential risk to aquatic ecosystems.

Key words: *Selenastrum capricornutum*; erythromycin lactobionate; ciprofloxacin hydrochloride; sulfamethoxazole; chlorophyll biosynthesis

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Introduction

Antibiotics are widely used in human disease therapeutics as well as in agricultural activities such as aquaculture and poultry farming. In the European Union (EU), 4700 tons of antibiotics are administered to farm animals and 8500 tons to humans in 1999 (FEDESA, 2003). In China, 15,770 tons of antibiotics were used as non-prescription therapeutics in 2004 (Richardson et al., 2005). The market share of macrolides antibiotics are the second largest in China, followed by quinolones antibiotics and sulfonamides antibiotics. Previous studies have shown that macrolides and sulfonamides antibiotics had the highest residue levels (e.g., 1–2 μg/L) in surface water, groundwater, and seawater (Lindsey et al., 2001; Ternes et al., 2002; Lalumera et al., 2004; Yang and Carlson, 2004; Richardson et al., 2005), and macrolides antibiotics residue reached 75 μg/L in some groundwater in Taiwan (Lin and Tsai, 2009). Therefore, the potential environmental effects upon non target organisms driven from widespread usage have attracted increasing attention, especially to primary producers in aquatic ecosystems. Studies on the toxic effects of antibiotics in aquatic environments has shown that the EC_{50} (72 hr) to *Selenastrum capricornutum* is 2.97 mg/L for ciprofloxacin, 7.8 mg/L for sulfadiazine, and 2.3 mg/L for spiramycin (Holten Ltzhφt et al., 1999; Halling-Sφensen, 2000).

Chlorophyll plays an important role in plant photosynthesis. Sufficient content and accurate localization are key to ensuring the photochemical reactions process. Koussevitzky et al. (2007) indicated that some antibiotics can interrupt chloroplast gene expression and inhibit chlorophyll synthesis. Bishop et al. (1973) found that chloramphenicol, as an antibiotic of a protein synthesis inhibitor, can interfere with chlorophyll biosynthesis significantly. But the effects of macrolides and quinolones antibiotics on chlorophyll biosynthesis in photosynthetic green algae remain largely unknown. Further studies are required to understand how these ecologically important freshwater microalgae respond physiologically to

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increased water-column antibiotic concentrations, especially to the exposure of extensively used antibiotics acting as inhibitors of protein synthesis like macrolides.

Green alga, *Selenastrum capricornutum* Printz, was selected to examine antibiotics toxicity. *Selenastrum capricornutum* is commonly used as a model organism of freshwater algae in standard toxicity tests (ISO 8692). Erythromycin, ciprofloxacin and sulfamethoxazole were the target compounds due to their widespread use in China. The objective of the present work was to study the toxic effects and toxic mechanisms of protein synthesis inhibited antibiotic (erythromycin) on chlorophyll biosynthesis through the measurement of chlorophyll content, seven important biosynthetic precursors contents and photosynthetic rate, compared with other groups of antibiotics (ciprofloxacin and sulfamethoxazole), and to evaluate potential ecological risks from antibiotics to aquatic environments via investigating the toxicological impacts of antibiotics to aquatic organisms.

1 Materials and methods

1.1 Algae culture

Selenastrum capricornutum was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, China. The composition of the growing medium was as follows: 3 mmol/L $NaNO_3$, 75 μmol/L KH_2PO_4, 1 mmol/L $NaHCO_3$, 300 μmol/L $MgSO_4{\cdot}7H_2O$, 250 μmol/L $CaCl_2{\cdot}2H_2O$, 30 μmol/L $FeCl_3{\cdot}6H_2O$, 46 μmol/L H_3BO_3, 9 μmol/L $MnCl_2{\cdot}4H_2O$, 0.8 μmol/L $ZnSO_4{\cdot}7H_2O$, 0.3 μmol/L $CuSO_4{\cdot}5H_2O$, 0.03 μmol/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (pH 5.8). Algae were cultured in an homoeothermic incubator with an illumination of 80 μmol/(m^2·sec) and under a light/dark period of 12 hr:12 hr, the temperature was set at 25°C (light)/20°C (dark). Each day, 0.1 mL of culture solution was collected and counted under a microscope to obtain alga cells density by haemocytometer.

1.2 Antibiotic treatment

Three types of antibiotics, erythromycin lactobionate (ETM), ciprofloxacin hydrochloride (CPF) and (SMZ) (purity > 98%) were purchased from Beta Pharma Ltd., Shanghai, China. Both ETM and CPF were dissolved by ultrapure water directly, with the stock solution concentration at 3 mg/mL. Sulfamethoxazole was dissolved in 20 mL of 0.1 mol/L NaOH, and then diluted with the ultrapure water to a final concentration of 3 mg/mL.

When the algae were at the logarithmic growth phase, individual antibiotics in the stock solution were added into algal medium at different concentrations: 0, 0.06, 0.12, 0.18, 0.24, 0.30 mg/L for ETM and 0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/L for CPF and SMZ. Algae were cultivated under the original conditions for 96 hr, and were then taken for the following physiological indices measurements. Three replications were prepared for each treatment. For each sample, alga cells in 100 mL culture solution were collected by filtration and the fresh weight was weighed. The quantity relations among the volume of culture solution and the fresh weight of alga cells were then acquired.

1.3 Determination of chloroplast pigments content

According to Wang (2006), after 96 hr of antibiotic exposure, 20 mL of culture solution was centrifuged at room temperature for 10 min at 7000 r/min. The collected algal pellet was suspended by 5 mL of 95% alcohol. The chloroplast pigments were extracted at 4°C for 24 hr in the dark and then centrifuged at 4°C for 10 min at 7000 r/min, the supernatant was analyzed spectrophotometrically at 470, 663 and 645 nm, respectively, then the concentration ($C_{\text{chl-}a}$, $C_{\text{chl-}b}$, C_{car}, mg/L) of chlorophyll *a* (chl-*a*), chlorophyll *b* (chl-*b*) and carotenoids (car) in the extracted solution were calculated according to Eqs. (1), (2) and (3), respectively.

$$C_{\text{chl-}a} = 13.95 \times \text{OD}_{665} - 6.88 \times \text{OD}_{649} \quad (1)$$

$$C_{\text{chl-}a} = 24.96 \times \text{OD}_{649} - 7.32 \times \text{OD}_{665} \quad (2)$$

$$C_{\text{car}} = 1000 \times \text{OD}_{470} - 2.05 \times C_{\text{chl-}a} - 114.8 \times C_{\text{chl-}b} \quad (3)$$

1.4 Chlorophyll biosynthetic main precursors assessment

Content of δ-aminolevulinic acid (ALA) was determined according to Dei (1985). Algae culture solution (200 mL) was centrifuged at 1000 ×*g* for 5 min and the algal pellet was collected, 200 mL of new sterilizing medium with 10 mmol/L of laevulinic acid was used to suspense algal cells, and then placed in an incubator under original culture conditions but light for 12 hr. Finally, algal culture was centrifuged to separate the algae. The ALA was extracted by using 4% trichloroacetic acid. The extracted solution (5 mL) was added with 2.35 mL of 1 mol/L sodium acetate, 0.15 mL of acetylacetonate and 2.5 mL of 1 mol/L acetate buffer (pH 4.6), heated in boiling water for 10 min, and finally Ehrlich reagent was added when the solution cooled down. The content of ALA was analyzed spectrophotometrically at 553 nm and calculated by the molar absorbency index at 553 nm, 7.2×10^4 L/(mol·cm).

Contents of porphobilinogen (PBG) were measured according to Bogorad (1962). Algal cells were collected from 200 mL of culture solution through centrifugation as mentioned above. The PBG was extracted by ultrasonication with the extracted buffer (pH 8.2) containing 0.1 mol/L EDTA and 0.6 mol/L Tris-HCl. Ehrlich reagent was then added. The content of PBG was determined spectrophotometrically at 553 nm, and calculated by the molar absorbance index at 553 nm, 6.1×10^4 L/(mol·cm).

As per Bogorad (1962), uroporphyrinogen III (Urogen III) and coproporphyrinogen III (Coprogen III) were extracted from the algal cells collected from 200 mL of culture solution by ultrasonication with phosphate buffer (pH 6.8). The extraction was added with 0.25 mL of 1% $Na_2S_2O_3$, and reacted for 20 min under high light intensity, adjusted to pH 3.5 by glacial acetic acid, and extracted by diethyl ether three times. The water-phase solution was then taken to analyze spectrophotometrically at 405.5 nm. The contents of Urogen III were calculated and expressed by the molar absorbance index at 405.5 nm, 5.48×10^5 L/(mol·cm). The above-mentioned solution extracted by

diethyl ether was re-extracted by hydrochloric acid three times. The hydrochloric acid phase was used to determine the contents of Coprogen III by spectrophotometer at 399.5 nm, the contents of Coprogen III was calculated by the molar absorbance index at 399.5 nm, 4.89×10^5 L/(mol·cm).

Contents of protoporphyrin IX (Proto IX), Mg-proporphyrin IX (Mg-Proto IX) and protochlorophyllide (Pchlide) were measured according to Rebeiz et al. (1975) and Lee et al. (1992). The *S. capricornutum* culture solution was placed in the dark for 12 hr, then extracted by ultrasonication with 8 mL of extraction solution (acetone : 0.1 mol/L $NH_3{\cdot}H_2O$ = 9:1, *V*/*V*) in the ice bath. The extracted solution were then re-extracted by equal-volume and 1/3 volume *n*-hexane in turn. Finally the acetone phase was taken to measure fluorescence intensity at 633 and 622 nm with 400 nm of excitation light and fluorescence intensity at 640 and 595 nm with 440 nm of excitation light by utilizing the LS 55 fluorescence/phosphorescence/luminescence spectrophotometer (PerkinElmer, USA). The relative content of Mg-Proto IX was represented by F_{595} directly (Lee et al., 1992); and the relative contents of Proto IX and Pchlide were calculated according to Eqs. (4) and (5) (Rebeiz et al., 1975).

$$\text{Proto IX} = (F_{633} - 0.25 \times F_{622} - 0.24 \times F_{640})/0.95 \quad (4)$$

$$\text{Pchlide} = (F_{640} - 0.03 \times F_{633})/0.99 \quad (5)$$

For each precursor content, data was normalized by the fresh weight.

1.5 Net photosynthetic rate assessment

After 96 hr exposure to antibiotic treatments, a portion of algae was taken for the determination of photosynthetic rate (Pn). Photosynthetic O_2 evolution was measured using a DW1 Oxygen Electrode Chamber (Hansatech Ltd., England) with an illumination of 300 μmol/(m^2·sec) red light intensity in 2 mL cuvette at room temperature. Each sample was measured continuously for 5 min. Data were normalized by the fresh weight.

2 Results

2.1 Alga growth

The growth of *S. capricornutum* during 96 hr in the presence of different antibiotic treatments is shown in Fig. 1. *S. capricornutum* was more sensitive to ETM than the other two antibiotics. It induced a significant inhibition effect on the growth rate at the concentration of 0.06 mg/L, while CPF and SMZ achieved the same effects at 1.5 and 2.0 mg/L, respectively (data not shown). The growth inhibition ratio of *S. capricornutum* at 96 hr were 65.9%, 30.8% and 25.9% under ETM, CPF and SMZ treatment, respectively.

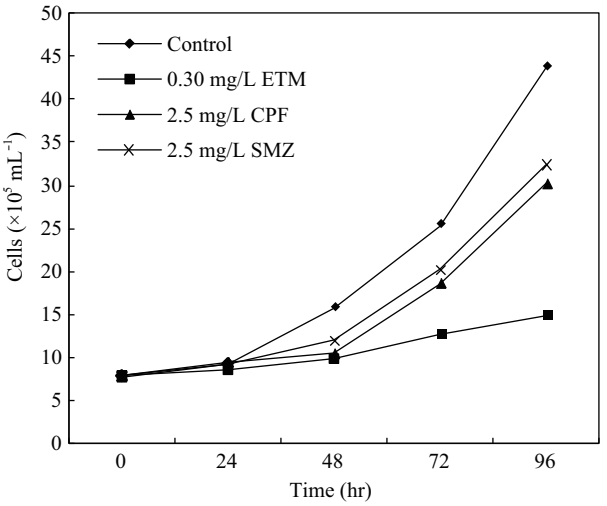

Fig. 1 *S. capricornutum* 96 hr growth in the absence and presence of different antibiotic treatments including 0.30 mg/L ETM, 2.5 mg/L CPF and 2.5 mg/L SMZ. Values are the mean of number of cells per mL culture solution in each treatment.

2.2 Chloroplast pigment content

The content of chl-*a* of *S. capricornutum* decreased significantly after exposure to ETM, and the effects increased with increasing ETM concentration (Fig. 2a). However, ETM had little effect on chl-*b*. The significant decrease was determined only when the concentration of ETM was > 0.18 mg/L. The same was true for carotenoid in ETM exposure. The content of carotenoid slightly decreased only when the concentration of ETM exposure was at 0.3 mg/L.

When the concentration of CPF treatment was higher than 2.0 mg/L, both chl-*a* and chl-*b* of *S. capricornutum* decreased significantly, but the level of this effect showed no obvious change with increasing CPF concentration. However, the carotenoid content began to decrease at 1.5 mg/L of CPF concentration, and the change level increased with increasing CPF concentration (Fig. 2b).

The content of chl-*a* in *S. capricornutum* was more sensitive to SMZ than CPF. It decreased significantly when the concentration of SMZ was at 1.5 mg/L, and decreased continuously with increasing concentration of SMZ. However, the effect of SMZ to the content of chl-*b* was similar to that of CPF, both led to a significant decrease in the content of chl-*b* at exposure concentration of 2.0 mg/L, but the level of this change was lower for SMZ. Carotenoid content decreased significantly when the concentration of SMZ was at 1.0 mg/L, but there was no obvious change with increasing SMZ concentration (Fig. 2c).

2.3 Chlorophyll biosynthesis

In terms of the measurement of the main precursors in the process of chlorophyll biosynthesis, including ALA, PBG, Urogen III, Coprogen III, Proto IX, Mg-Proto IX and Pchlide, we found that all chlorophyll biosynthesis precursors in *S. capricornutum* were reduced significantly after ETM exposure, and the level increased with increasing ETM concentration (Fig. 3a). However, the effect of CPF to the chlorophyll biosynthesis precursors content

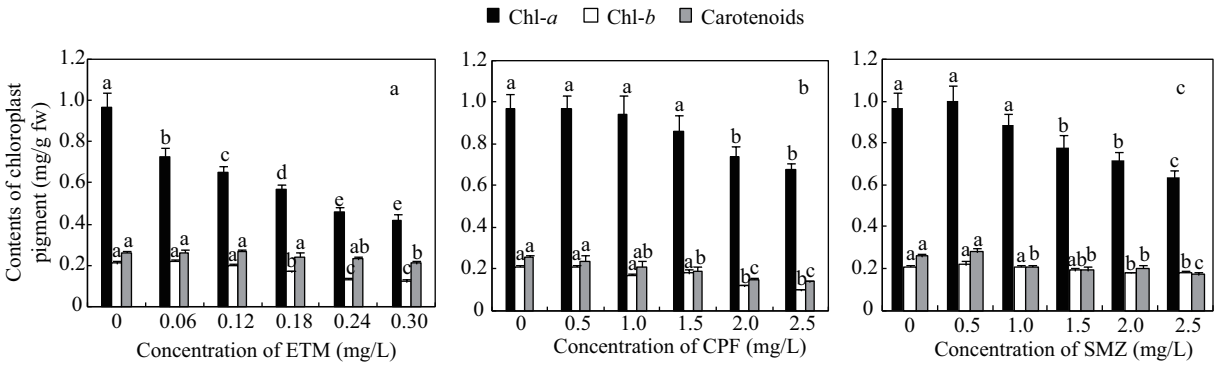

Fig. 2 Contents of chlorophyll *a*, chlorophyll *b* and carotenoids of *S. capricornutum* in control and antibiotic treatment groups including ETM (a), CPF (b) and SMZ (c). Bars indicate standard deviation (SD) of the mean (n=3). Significant differences from the controls are represented by letters.

of *S. capricornutum* was smaller. With the exception of Proto IX and Pchlide, all precursors decreased slightly only when the concentration of CPF was higher than 2.0 mg/L (Fig. 3b). However, no obvious change in precursor content was observed in the SMZ treatment (Fig. 3c).

2.4 Photosynthetic rate

Net photosynthetic rate (Pn) of *S. capricornutum* was very sensitive to ETM exposure. Pn decreased with increasing ETM concentration (Fig. 4a). Pn was less than 25% of the control value when ETM treatment concentration was at 0.3 mg/L.

Upon exposure to CPF and SMZ, Pn decrease significantly at concentrations of 1.0 mg/L and 0.5 mg/L, respectively (Fig. 4b, c), but the degree of Pn reduction in CPF and SMZ exposures were much smaller than that in ETM exposure. When the concentration of SMZ treatment was higher than 2.0 mg/L, Pn did not further decrease.

3 Discussion

Alga has different sensitivities to different antibiotics. In the present study, ETM exhibited more toxic effect on the growth of *S. capricornutum* than CPF and SMZ, which accords with conclusions of previous studies (Halling-Sϕrensen, 2000; Holten Lützhϕt et al., 1999). The possible explanation for this discrepancy may be that the genetic expression in chloroplast was much similar to prokaryotes (Halling-Sϕrensen, 2000), therefore macrolides such as ETM could directly induce the inhibition of many functional proteins encoded by chloroplast genes and interfer with many physiological processes, including chlorophyll biosynthesis and photosynthesis.

Chlorophyll biosynthesis is a complicated plant process comprised of 16 reactions catalyzed by 16 different enzymes, which are encoded by more than 20 genes (Beale, 2005). The total process initiated by Glutamyl-tRNA, and produced seven main precursors: ALA, PBG, Urogen III, Coprogen III, Proto IX, Mg-Proto IX and Pchlide, and finally synthesized chl-*a* and chl-*b* (Reinbothe and Reinbo, 1996; Brusslan and Peterson, 2002).

Chlorophyll plays an important role in energy capture and transfer during photosynthesis. Xiao et al. (2010) found that some organic contaminants can significantly decrease chlorophyll pigment content in *Phaeodactylum tricornutum*. In addition, Bishop et al. (1973) found that chloramphenicol significantly inhibited chlorophyll biosynthesis in *Euglena gracilis*. Both ETM and chloramphenicol belong to protein synthesis inhibited antibiotics, so ETM should have similar effects as chloramphenicol, as was demonstrated in the present study. The content of the seven precursors in chlorophyll synthesis significantly decreased under 0.06 mg/L of ETM exposure. This indicated that ETM inhibited chlorophyll biosynthesis of *S. capricornutum* from the first step at low concentration.

Sixteen enzymes used in plant chlorophyll biosynthesis are encoded by nuclear genes (Beale, 2005), therefore ETM should not have any direct inhibitive effects on the synthetic process of these enzymes. However, many protein complexes are co-encoded by both chloroplast and nuclear genes, so the corresponding expression between two genomes is very important for chlorophyll biosynthesis (Larkin et al., 2003; Koussevitzky et al., 2007; von Gromoff et al., 2008). Some thylakoid membrane proteins, such as the chlorophyll *a/b* binding antenna protein related to the correct localization of chlorophyll, are encoded by the chloroplast gene (Dyer, 1985), and ETM could interfere with the localization process of chlorophyll via its inhibition of the synthetic process of these proteins. Koussevitzky et al. (2007) found that the expression of the nuclear gene of plants related to chlorophyll synthesis can be inhibited by some feedback models in a series of signal pathways when chloroplast gene expression was inhibited. Larkin et al. (2003) suggested some precursors of chlorophyll biosynthesis can also inhibit nuclear gene expression by some signal pathways in their study of *Arabidopsis thaliana*. ETM can interfere with chlorophyll biosynthesis via the inhibition of chloroplast gene expression, and then further affect nuclear gene expression indirectly.

The effects of CPF and SMZ to the content of chlorophyll biosynthetic precursors in *S. capricornutum* were much weaker than that of ETM, but they induced a decrease in chlorophyll contents at high exposure concentrations. It has been reported that changes in the redox state of the photosynthetic electron transport chain can affect nuclear gene expression and interfere with the localization of chlorophyll (Koussevitzky et al., 2007). High concentrations of CPF and SMZ may influence the redox state of the photosynthetic electron transport chain by free radicals accumulated via a series of redox reactions (Liu et al.,

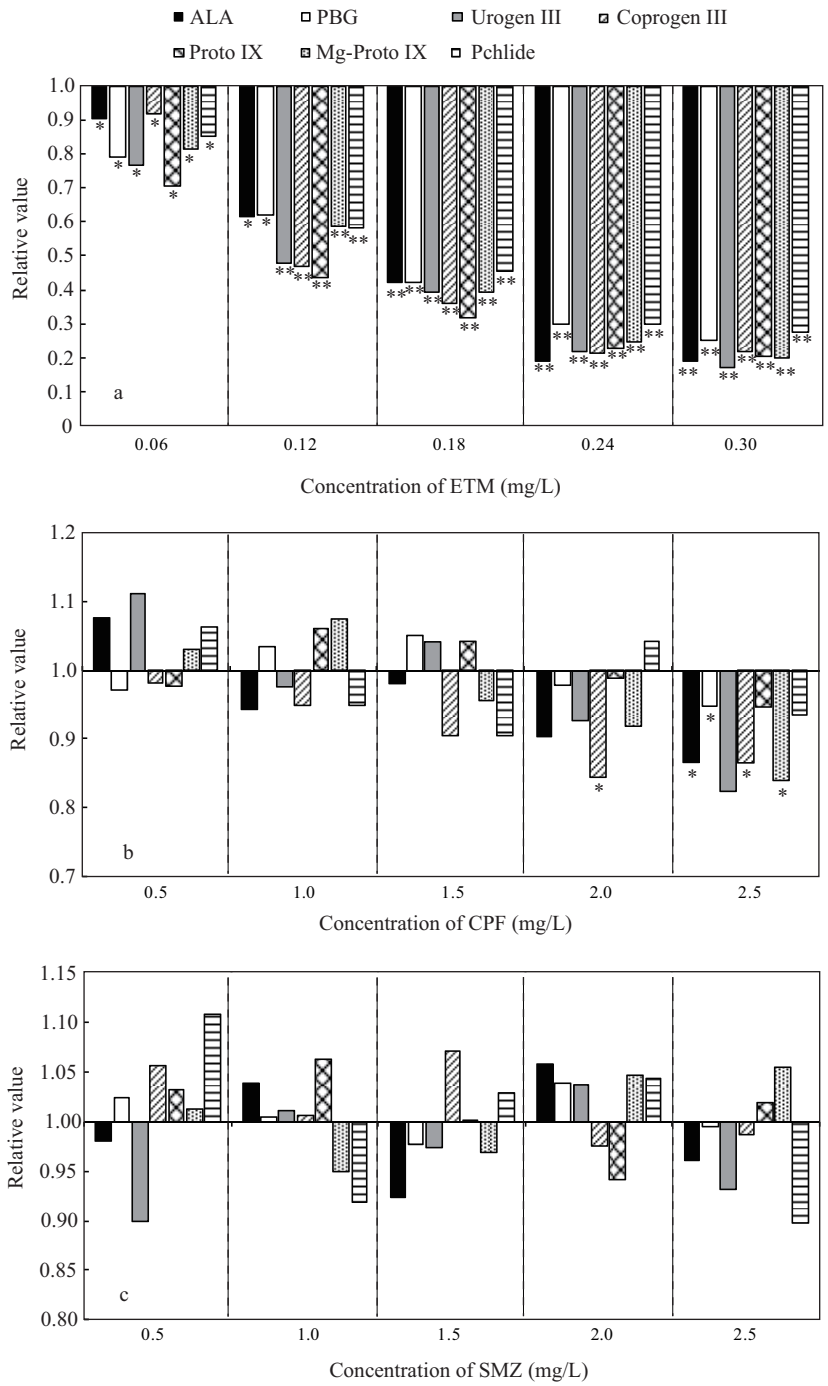

Fig. 3 Contents of chlorophyll biosynthetic main precursors included δ-aminolevulinic acid (ALA), porphobilinogen (PBG), uroporphyrinogen III (Urogen III), coproporphyrinogen III (Coprogen III), protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX) and protochlorophyllide (Pchlide) in *S. capricornutum* after antibiotic treatments including ETM (a), CPF (b) and SMZ (c). The data have been normalized to the control as 1. Significant differences from the controls are represented by * ($p < 0.05$) and ** ($p < 0.01$).

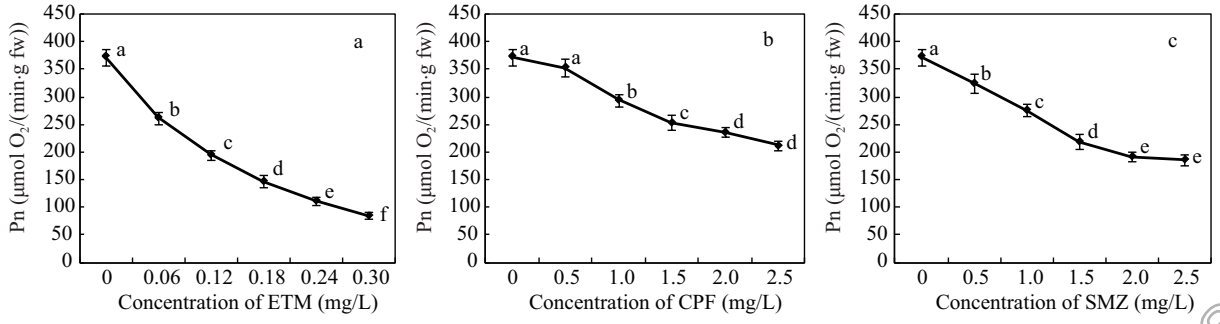

Fig. 4 Net photosynthesis rate (Pn) of *S. capricornutum* after antibiotic treatments including ETM (a), CPF (b) and SMZ (c). Bars indicated standard deviation of the mean ($n = 3$). Significant differences from the controls are represented by different letters ($p < 0.05$, one-way ANOVA).

2011), then induced the chlorophyll could not be localized correctly, and finally, led to the decrease of chlorophyll contents due to the degradation of the free-state chlorophyll in the chloroplast. In addition, CPF can inhibit DNA topoisomerase activity and interfere with DNA replication, consequently reduce the quantity of chloroplast (Krajčvič and Ebringer, 1990), and finally, lead to the decrease in the content of chlorophyll.

Some previous studies have suggested that tetracycline can inhibit the activity of chlorophyll synthase, and then induce a decrease in chlorophyll content (Bradel et al., 2000). But further studies are still required to determine whether ETM, CPF and SMZ have a similar effect.

Photosynthetic apparatus can be significantly affected by some heavy metal ions and organic contaminants (Hattab et al., 2009; Xiao et al., 2010). The decrease of *S. capricornutum* Pn in response to all three types of antibiotics exposure suggested that they all obviously inhibited the photosynthesis of *S. capricornutum*. Among these antibiotics, the toxic effects on the photosynthesis of *S. capricornutum* were stronger for ETM compared to CPF and SMZ. In addition, ETM displayed obvious inhibition effects on the synthesis of some photosynthesis related proteins (Liu et al., 2011), so the decreased Pn induced by ETM may be due to their inhibition effects on the biosynthesis of some important substances such as chlorophyll. In comparison, the effects of CPF and SMZ on chlorophyll biosynthesis were not significant, so decreasing Pn induced by CPF and SMZ may be related to their inhibition effect on photosynthetic electron transport (Liu et al., 2011). It is considered that carotenoids are antioxidants *in vivo*, to quench the intra-cellular free radicals (Thuenhan, 1990). Many organic pollutants can induce free radical accumulation through a series of oxidation reactions. Organic contaminants can result in changes in antioxidant enzyme activity in plants, which is related to free radical accumulation due to antibiotics exposure (Roy et al., 1995). This may explain the changes in carotenoid content after antibiotic exposure.

Acknowledgments

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