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RESEARCH ARTICLE

Toxic effects of enrofloxacin on *Scenedesmus obliquus*

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Abstract In this article, the toxic effects of Enrofloxacin (ENFX) on *Scenedesmus obliquus* were studied, through investigating the growth, photosynthetic pigments, and protein contents. The possible toxic mechanisms of ENFX were analyzed by determining the superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, proline content, and superoxide anion (O_2^-) generation rate. Results showed that the growth of algae was inhibited by ENFX and the 50% effective concentration (EC_{50}) values for 24, 48, 72, and 96 h of ENFX were 88.39, 63.86, 45.10, and 59.16 $mg \cdot L^{-1}$, respectively. After treated with ENFX for 96 h, the contents of photosynthetic pigments decreased with the increase of ENFX concentration, the content of soluble protein and the activity of SOD increased and then decreased, and the generation rate of superoxide anion (O_2^-) increased continually. The contents of MDA and proline changed little in lower ENFX concentration groups, but increased rapidly when treated with higher concentration groups. These results suggested that ENFX affected the growth of *S. obliquus*, and the main toxicity mechanism was that algal cells generated the reactive oxygen species under ENFX stress, and then the reactive oxygen species (ROS) induced the oxidation damages of biologic macromolecules and changed the biomembrane permeability further.

Keywords enrofloxacin, *Scenedesmus obliquus*, toxic effects

1 Introduction

In recent years, with the analytical techniques becoming more sensitive and more widely applied, an increasing number of pharmaceutical ingredients are being detected in aquatic environment in their parent form or as metabolites. They are all at concentrations in the nanogram per liter to microgram per liter range [1–5]. This has prompted public and scientific concerns [6], and also inspired a great interest in this research area, which focused on the sources, occurrence, fate, and routes of exposure of these compounds in the environment [7–10]. However, little is known about their potential adverse effects on aquatic organisms and ecosystems, despite their ubiquitous occurrence and high biologic activity [5].

Enrofloxacin (ENFX) is a fluoroquinolone group antibiotic, and it is widely used in poultry production, animal husbandry, and aquaculture for treating respiratory and enteric bacterial infections. This antibiotic acts directly on bacterial DNA by inhibiting cell reproduction which leads to cell death. After used, ENFX will be excreted in urine or faeces as a mixture of unchanged parent compound or metabolites [7], and then enter the sewerage system and pass through sewage treatment before release via sludge or effluent discharge to surface waters. Recent studies indicate that antibiotics used in aquaculture are directly added to receiving waters or formulated as feed additives. Most of the antibiotics are water-soluble and therefore about 90% of one dose can be excreted in urine and up to 75% in animal faeces, leading that 70%–80% of administered drug will enter the environment [11,12]. When the antibiotics enter the aquatic environment, they will not only affect the target organisms, but also may pose a potential threat to non-target aquatic lives and aquatic ecosystems [13].

Algae, as the primary producer in the aquatic ecosystems, are of great importance in assessing the water environmental quality. Moreover, the species diversity and primary production play a vital role for the balance of the ecosystems. Therefore, algae are frequently used in various bioassays. *Scenedesmus obliquus* (*S. obliquus*) as one of the standard test algae in algale growth inhibition test recommended by Organization for Economic Cooperation and Development (OECD) is sensitive to toxic compounds, easy to cultivate, rapid in reproduction and its response is highly reproducible. Toxic compound may

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affect microalgal growth, respiration, photosynthesis, and enzyme activity [14]. The effective concentration of toxicants that inhibits 50% microalgal growth at 96 h (96 h EC_{50}) is widely used as an index of toxicity.

In this paper, the toxic effects of ENFX on *S. obliquus* were studied, through investigating the algal growth, the photosynthetic pigments and protein contents, the superoxide dismutase (SOD) activity, the malondialdehyde (MDA) content, the proline content, and the superoxide anion (O_2^-) generation rate. It is expected that the possible toxic mechanisms of ENFX on *S. obliquus* will be found out, and the results can provide the scientific evidence for the reasonable use and water environmental risk assessment of ENFX.

2 Materials and methods

2.1 Plant material and chemicals

S. obliquus was obtained from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences, cultivated in 100 mL liquid Soil Extract medium prepared in accordance with the China State Environmental Protection Bureau Guidelines 201, as reported by Chen et al. [15], in 250 mL flasks, and illuminated at 4000 lx light intensity with a light:dark cycle of 12:12 [16]. Temperature was maintained in an air-conditioned growth chamber (LRH-300-Gb, Shaoguan Xinteng, China) at 25°C±1°C. Cells in the exponential phase of growth were collected from stock cultures and used for experiments as the inocula. After inoculated in the medium, every 24 h, certain volume algae cells were taken and optical densities namely absorbance were analyzed at 650 nm light wavelength. Also, algae cell numbers were counted using Petroff-Hausser counting chamber (XB-K-25, Yancheng Technical, China) under an optical microscope (YS2-H, Nikon, China), then the standard curve of algae cell density and optical density was established.

Enrofloxacin hydrochloride (analytical grade, Zhejiang Guobang Pharmaceutical Co., Ltd., China). And ENFX stock solution is prepared by dissolving this compound in test medium before the test. Acetone (99.5%), Hydroxylammonium chloride (98.5%), Phosphoric acid (85%) and Triethylamine (99%) were purchased from Beijing Chemical Works, China. Trichloroacetic acid (TDA, 99%), and 2-Thiobarbituric acid (98.5%) were from Sinopharm Chemical Reagent Co., Ltd., China. Coomassie brilliant blue G-250, Nitroblue tetrazolium (98%), and Sulfosalicylic acid (99%) were supplied by Shanghai Huishi Biochemical Reagent Co., Ltd., China. Acetonitrile was chromatogram grade and from Dima Technology Inc, USA. Chemicals used for growth medium were all of analytical grade and purchased from Beijing Chemical Works, China.

2.2 Toxicant treatment

For the toxicity assays, at the beginning of the experiment, different volumes of ENFX stock solution were diluted in the culture medium which has been sterilized at 121°C for 30 min, so the ENFX concentrations were at 0, 24, 48, 80, 96, and 120 $mg \cdot L^{-1}$, respectively. The ENFX concentrations decreased slightly with the exposure time, and the concentration decrease during the tests did not exceed 6%. The highest decrease was observed for 80 $mg \cdot L^{-1}$ group at 96 h, about 5.33%. The ENFX concentration was measured with a high pressure liquid chromatography (HPLC) (LC-20A, Shimadzu, Japan) according to the procedure and the conditions described by Ministry of Agriculture of China [17]. Both control and test flasks were inoculated with exponential growing algae so an initial concentration of *S. obliquus* was 3×10^5 $cells \cdot mL^{-1}$. Each test concentration was replicated three times, and all operations were carried out under sterile conditions to avoid contamination from bacteria.

2.3 Growth inhibition and EC_{50} determination

From 0 to 96 h, 3 mL algal cells were taken at 0, 24, 48, 72, and 96 h and optical density were determined by ultraviolet and visible (UV-Vis) spectrophotometer (UV-190, Shimadzu, Japan) at 650 nm light wavelength in 1 cm colorimetric utensil. Then algae cell numbers were calculated according to the standard curve to represent the biomass, at last the growth rate and inhibition percentage were calculated according to the equation as follows [18]:

$$V_t = \ln(N_t/N_0)/(t-t_0), \quad (1)$$

$$\%I = (V_n - V_t)/V_n \times 100, \quad (2)$$

where N_t is the cell number at t time, N_0 is the cell number at 0 time, t is the sample exposure time, and t_0 is the origin time of the treatment, V_t and V_n are the average specific growth rates in the presence of ENFX and in the control, $\%I$ is the percent inhibition in average specific growth rate.

According to the correlation of the percentages of growth inhibition and the concentration data, the exact EC_{50} values at 24, 48, 72, and 96 h were calculated.

2.4 Main physiologic and biochemical indexes determination

To determine the effects of ENFX on the photosynthetic pigments contents, 10 mL algal cells suspensions were transferred into centrifuge tubes after *S. obliquus* was exposed for 96 h, and centrifuged at 4000 $r \cdot min^{-1}$ for 10 min. The supernatant was discarded and 5 mL 80% acetone was added, and then mixed well. The above

mixture was settled in the dark for 24 h to extract. The extracts were then centrifuged at 10000 r·min^{-1} for 10 min. The supernatant was analyzed for optical density at 663, 645, and 470 nm light wavelength in 1 cm colorimetric utensil. The photosynthetic pigments contents were calculated as [19]

$$\text{C-a} = 12.21\text{OD}_{663} - 2.81\text{OD}_{645}, \quad (3)$$

$$\text{C-b} = 20.13\text{OD}_{645} - 5.03\text{OD}_{663}, \quad (4)$$

$$\text{C-k} = (1000\text{OD}_{470} - 3.27\text{C-a} - 104\text{C-b})/229, \quad (5)$$

where C-a is the content of chlorophyll-a (mg·L^{-1}), C-b is the content of chlorophyll-b, and C-k is the content of carotenoids.

At the end of the exposure (96 h), 100 mL algae cells suspensions were collected and centrifuged at 4500 r·min^{-1} for 10 min, then 10 mL of phosphate buffer (0.05 mol·L^{-1}, pH 7.8) and small amount of silica sand were added, and ground in ice bath. The slurry was centrifuged at 10000 r·min^{-1} for 15 min. The content of protein in the supernatant was determined by Coomassie Brilliant Blue method [20], the SOD activity was determined by Nitroblue Tetrazolium Photoreduction [20], and the O_2^- generation rate was studied using hydroxylamine oxidization method [21]. The collected algae cells was mixed with 10 mL 10% trichloroacetic acid and small amount of Silica Sand, ground in ice bath. The slurry was centrifuged at 10000 r·min^{-1} for 15 min, 4°C temperature. Then thiobarbituric acid method was used to determine the content of MDA [20]; 5 mL 3% sulfosalicylic acid was added into the collected algae cells, then the mixture was extracted in boiling water bath for 30 min and filtered into a clean test tube after cooling. The filtrate was the proline extract and the proline content was determined by spectrophotometer [20].

2.5 Statistical analysis

The experimental data were analyzed with Statistical Package for the Social Sciences (SPSS) software (version 13.0, SPSS Inc., USA) and Minitab software (version 14, Minitab Inc., USA), and significant differences among treatments were statistically analyzed using one-way analysis of variance ($P < 0.05$, significant difference; $P < 0.01$, extremely significant difference).

3 Results and discussion

3.1 Standard curve of algae cell density and optical density

The regression equation of algae cell density (Y) and optical density (X) was $Y = 11.037X - 0.0926$ (Fig. 1), which counted using Petroff-Hausser counting chamber under an optical microscope and analyzed at 650 nm with a spectrophotometer, and the coefficient of correlation R was 0.998. Then the growth of algae and biomass were calculated by measurement of OD_{650}.

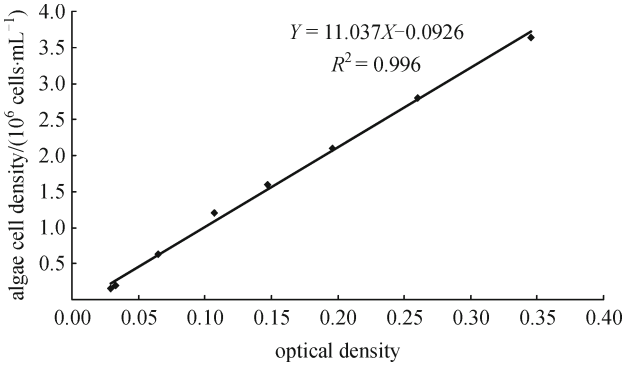

Fig. 1 Relationship between cell density of *S. obliquus* and optical density

3.2 Effects of ENFX exposure on the growth of *S.obliquus*

Figure 2 shows the growth curve of *S. obliquus* cultured in different concentrations of ENFX. It can be found that ENFX could inhibit the algal growth, and the algal growth inhibition increased with ENFX exposure concentration increasing, which could caused by that in lower concentrations treatment, individual cells was relatively lower load of antibiotic, *S. obliquus* cells were still able to divide rapidly, and the growth rates were not as that in the control group but still steady growth; in higher treatment groups, the concentration of ENFX might be over the endurance capability of the algae cells, the growth of algae cells was inhibited, but not appeared zero growth or negative growth. At 72 h exposured, the growth inhibition was the most significant. For exposure concentration 24, 48, 80, 96, and 120 mg·L^{-1}, the growth inhibition were 27.27%, 50.00%, 72.72%, 77.27%, and 90.91%, respectively. The growth of *S. obliquus* was inhibited seriously.

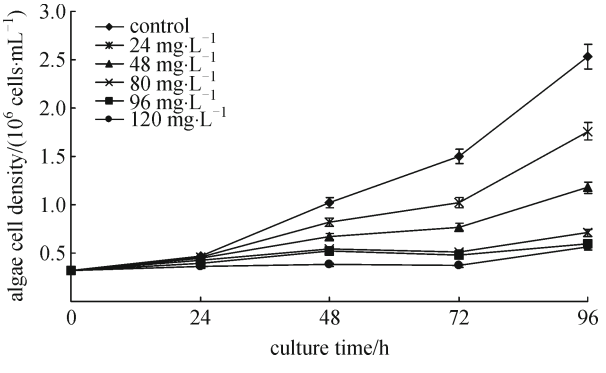

Fig. 2 Growth curve of *S. obliquus* in different concentrations of ENFX

According to the data from Fig. 2, through the statistical analysis, the EC_{50} value of different intervals could be calculated. The 24, 48, 72, and 96 h EC_{50} for ENFX were 88.39, 63.86, 45.10, and 59.16 $mg \cdot L^{-1}$, respectively (Table 1), while in another report the 24 and 48 h EC_{50} of ENFX were 119.67 and 152.59 $mg \cdot L^{-1}$ to *Haematococcus pluvialis* [22]. This might be caused by the difference of the various species sensitivities. Also an overview of the toxicity data of other quinolone antibiotics to algae, found in the literature, is shown in Table 2. From the toxicity data established in this investigation as well as data found in the literature, it is realized that among algae, cyanobacteria have shown to be the most sensitive algal species, due to their structure being more like bacteria. Also it was shown that quinolone antibiotics exhibited the same toxic pattern, namely being toxic, quantified as EC_{50}, in the few $mg \cdot L^{-1}$ range to green algae. And ENFX might be one of the quinolone antibiotics who have the less toxicity to algae.

The regression equations, correlation coefficients (R) and 95% confidence intervals could be found in Table 1. From the table, it can be seen that the toxicity of ENFX to *S.obliquus* increased and then decreased when the exposure time was over 72 h, so this suggested that at the beginning, the resistance of algae itself reduced and a slower recovery, when the exposure time was more than 72 h recovered partly and rapidly.

3.3 Effects of ENFX exposure on the main physiologic and biochemical indexes of *S.obliquus*

3.3.1 Effects on photosynthetic pigments contents

As with higher plants, algal photosynthetic pigments includes chlorophyll-a, chlorophyll-b, and carotenoids, and in all phytoplankton, chlorophyll-a which is the material basis of photosynthesis of algae accounted for about 1%–2% in organic dry weight. The effect of ENFX on photosynthetic pigments contents is shown in Fig. 3. It was obvious that ENFX affected photosynthetic pigments contents in *S.obliquus*. Significant reductions were found in photosynthetic parameters with all ENFX treatments. Total chlorophyll content decreased with increasing ENFX concentrations. When ENFX concentration was greater than or equal to 80 $mg \cdot L^{-1}$ in the treatment groups, the chlorophyll-a contents were extremely significantly different ($p < 0.01$), decreased to 16.59%, 15.28%, and 5.66%, respectively, compared to the control group. At the concentration of ENFX in 120 $mg \cdot L^{-1}$, the contents of

Table 1 Acute toxicity of ENFX to *S. obliquus*

culture time/h	regression equation [a)]	$EC_{50}/(mg \cdot L^{-1})$	R	95% confidence interval
24	$Y = 38.49X - 124.90$	88.39	0.913	53.29–146.61
48	$Y = 37.21X - 104.66$	63.86	0.964	48.19–84.61
72	$Y = 38.89X - 97.93$	45.10	0.996	40.20–50.60
96	$Y = 35.40X - 94.43$	59.16	0.996	53.58–65.31

Note: a) Y and X stand for percentage inhibition ($0 \leqslant Y \leqslant 100$) and natural logarithm of ENFX concentration, respectively

Table 2 Overview of the toxicity data of quinolone antibiotics to algae

compound	algal species	$EC_{50}/(mg \cdot L^{-1})$	references
ciprofloxacin	*Microcystis aeruginosa*	0.005	[23]
	Selenastrum capricornutum	2.97	
	Chlorella vulgarrs	20.61	[24]
flumequin	*Microcystis aeruginosa*	0.159	[23]
	Selenastrum capricornutum	5	
	Rhodomonas salina	18	
oxolinic acid	*Microcystis aeruginosa*	0.18	
	Selenastrum capricornutum	16	
	Rhodomonas salina	10	
sarafloxacin	*Microcystis aeruginosa*	0.015^{OMR}	
	Selenastrum capricornutum	16	
	Rhodomonas salina	24	
norfloxacin	*Chlorella pyrenoidosa*	30.78	[25]
	Scenedesmus obliquus	50.18	[26]

Note: OMR: out of measured range

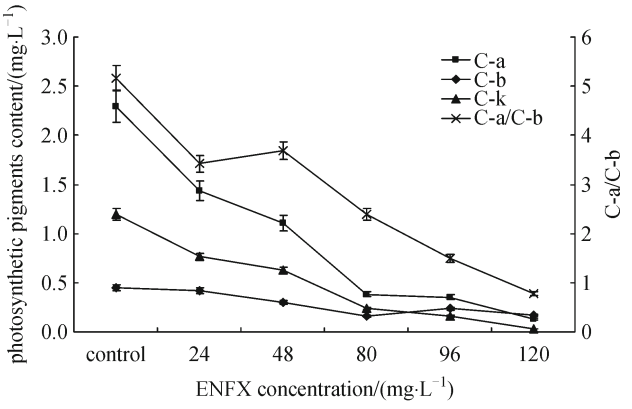

Fig. 3 Effects of different concentrations of ENFX on photosynthetic pigments contents of *S. obliquus* (96 h exposure)

chlorophyll-b and carotenoids declined to 37.75% ($P < 0.01$) and 2.59% ($P < 0.01$), respectively. It can also be seen from the figure, chlorophyll-b and carotenoids contents were much lower than chlorophyll-a in each test group, and changes in chlorophyll-a could better reflect the degree of algal impairment. Because the changes of chlorophyll contents took place at the molecular level in cells, they happened much earlier than growth [27]. Therefore, chlorophyll-a could be used as a more sensitive parameter than the growth inhibition for early warning of antibiotic exposure. And chlorophyll-a: chlorophyll-b ratio in *S.obliquus* was 5.16, 3.43, 3.69, 2.39, 1.49, and 0.78, suggesting that the structure of chlorophyll body could be affected by ENFX. The decrease in the ratio of chlorophyll-a: chlorophyll-b has been linked with the change in pigment composition of photosynthetic apparatus which possesses lower level of light harvesting chlorophyll proteins (LHCPs) [28]. Meanwhile microscopic examination showed that individual algae cells wall was damaged, algae cells decomposition could occurred, and algae liquid changed white. These might be caused by 1) antibiotic stress caused the chloroplast swelling and rupture, thylakoid membrane disintegration, thus resulting in the loss of chloroplasts from tissue [29], or 2) the accumulation of intracellular reactive oxygen species which would lead to the destruction of chloroplast structure, chlorophyll synthesis was blocked [30,31], or 3) the antibiotic directly combined with certain algal ingredients, this led to the inhibition of light harvesting chlorophyll-a/b proteins complex synthesis in chloroplast lamella [32].

3.3.2 Effects on protein content

Protein is an important structural material in organisms, and it participates in virtually every process of metabolism within cells as a catalyst. Soluble protein in organisms plays an important role as an indicator of reversible and irreversible changes in metabolism, and is known to respond to a wide variety of stressor such as natural and xenobiotic [33]. Reinheckel et al. reported that the functionality of protein can be affected by reactive oxygen species (ROS) either by oxidation of amino acid side chains or by secondary reactions with aldehydic products of lipid peroxidation [34]. After 96 h treatment with different concentrations of ENFX, the content of soluble protein in *S. obliquus* cells increased and then decreased (Fig. 4). Protein contents in each treatment group were significantly different with the control group ($p < 0.05$), the 48, 80, and 120 mg·L^{-1} treatment groups were extremely significantly different ($P < 0.01$). When the treating concentration was 80 mg·L^{-1}, the soluble protein content was 173% of the control; however, the content was the lowest at 120 mg·L^{-1} treating concentration, only 33.05% in comparison to the control. Under adversity stress, the protein synthesis was inhibited, the stress response occurred in plant to stabilize the intracellular enzyme system through inducing the synthesis and accumulation of new proteins, and also for increasing the content of protein [35]. Meanwhile, the endogenous protein might play a role in scavenging reactive oxygen species [36]. Under ENFX stress, the change of protein content in algae cells that increased at first and then decreased may be because algae cells changed the gene expression, that is to say, closed some normal expressed genes and initiated the genes that adapted to the stress, thus synthesized new antioxidant enzymes or isozymes which have more stable structures and more powerful functions, in order to compensate the damages of antioxidant enzymes caused by oxygen free radicals. The change of gene expression resulted in the increase of the protein content. When ENFX concentration was above 80 mg·L^{-1}, the protein content decreased rapidly, indicated that ENFX-induced oxidative stress appeared obvious in *S.obliquus*, which probably because more ENFX entered into algal cells, and this induced the excessive production of oxygen

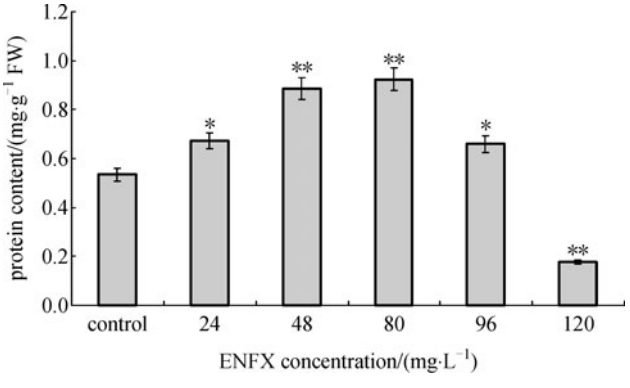

Fig. 4 Effects of different concentrations of ENFX on soluble protein of *S. obliquus* (96 h exposure). *, **: compared with the control group, $p < 0.05$, $p < 0.01$; FW: fresh weight

free radicals, so the original balance was destroyed, resulted in the metabolic disorder. The irreversible damage occurred in cell structure, even in the body of protein, thus inhibited the formation and accumulation of the soluble protein in *S. obliquus*.

3.3.3 Effects on SOD activity and O_2^- generation rate

Some published reports demonstrated that one of the major consequences of the compound's toxicity, such as fungicides triadimefon (TDM) and hexaconazole (HEX), should be the enhanced production of ROS [37–39]. The ROS include the superoxide anion radical (O_2^-•), hydroxyl radical (HO•), and the hydrogen peroxide (H_2O_2) which are produced as by-products during membrane linked electron transport activities as well as by a number of metabolic pathways and then cause damage to the biomolecules such as membrane lipids, protein, chloroplast pigments, enzymes, carbohydrates, and nucleic acids [40,41]. It seems that plants possess a powerful defense system to protect the photosynthetic apparatus and cellular membranes from ROS, thereby prevent oxidative damage. The production of antioxidative enzymes would be one part of the defense system which can protect plants against adversity stress. SOD is a family of antioxidative enzymes that act as a first cell defense against oxidative stress to eliminate ROS or reduce damaging effects. And the balance between ROS generation and eradication determines the survival of the system. Thus the changes of the SOD activity and O_2^- generation rate may reflect the degree of plants under adverse stress. In the present study, the effects of ENFX on SOD activity are shown in Fig. 5. It showed that the O_2^- generation rate increased with the increase of the ENFX concentration. The rate of 24 $mg \cdot L^{-1}$ group was not much different from the control. When ENFX concentration was greater than or equal to 48 $mg \cdot L^{-1}$, the O_2^- generation rate reached a extremely significant difference ($p < 0.01$), compared the control, which indicated more O_2^- was generated under ENFX stress at higher concentrations. When *S. obliquus* was exposed at the highest concentration (120 $mg \cdot L^{-1}$), the rate of O_2^- generation reached 2.57 folds of the control group ($p < 0.01$). Meanwhile, the SOD activity increased and then decreased with the increase of ENFX exposure concentration. The SOD activities in each treatment group were extremely significantly different ($p < 0.01$) from the control group. When the exposure concentration was less than or equal to 48 $mg \cdot L^{-1}$, the activity of SOD increased more slowly. The maximum stimulation of SOD activity (1.53-fold) was observed when algae cells were exposed to 80 $mg \cdot L^{-1}$ for 96 h duration. With increasing exposure concentration, the SOD activity decreased, especially at an exposure concentration of 120 $mg \cdot L^{-1}$, the SOD activity decreased sharply, and the minimum SOD activity was observed only 77.55% of the control. The changes of SOD activity was most likely that the presence of ENFX at lower concentration stimulated the O_2^- generation, thereby induced the increase of SOD activity, which catalyzed the conversion of O_2^- to O_2 and H_2O_2 [42]. When exposed to a higher concentration (above 80 $mg \cdot L^{-1}$), the SOD activity declined with the increase in ENFX levels, and this might be the result of an indirect effect mediated via a great increase in levels of O_2^- radicals or its poisonous active oxygen derivatives [43]. Then the inhibition of SOD failed to scavenge O_2^- to protect plant such as algae from cellular oxidative damage which included the membrane lipids, protein and chloroplast pigments oxidative damage, since O_2^- acted as a precursor of more cytotoxic or highly reactive oxygen derivatives [44]. Also the decrease of activity of SOD would lead to generate more O_2^-.

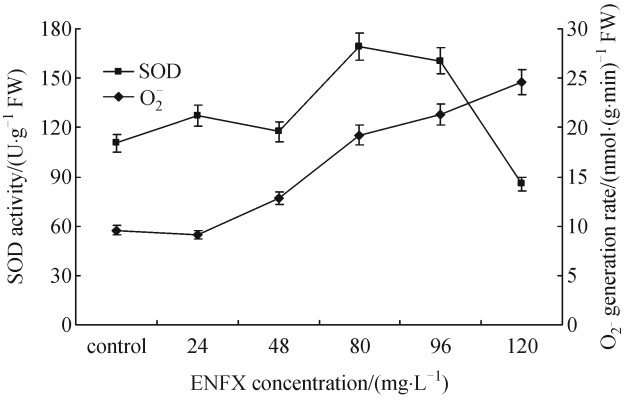

Fig. 5 Effects of ENFX on SOD activity and O_2^- generation rate of *S. obliquus* (96 h exposure). FW: fresh weight

3.3.4 Effects on MDA content

When plants are under stress, the generation and accumulation of reactive oxygen species may occur and lead to lipid peroxidation. Also the determination of Malondialdehyde levels is the most commonly applied assay for lipid peroxidation in biomedical sciences, since MDA is one of the better-known decomposition products of lipid peroxidation in polysaturated fatty acids of biomembranes [45,46]. Therefore, it is considered a good biomarker of free radical damage to lipids in biomaterials, even used as an indicator of cell membrane injury. In this study, ENFX treatment also enhanced lipid peroxidation in algal cells, as indicated by the increased MDA content in algal cells homogenate (Fig. 6). It showed that the MDA contents of all the ENFX-treated cultures were much higher than those of the control, increased with the increase of ENFX concentration. When the treatment concentration was greater than or equal to 80 $mg \cdot L^{-1}$, the MDA content was significant different ($p < 0.05$) from that of the controls. Even the stimulation of oxidative damage caused by lipid peroxidation 105.76% and 145.67% higher than

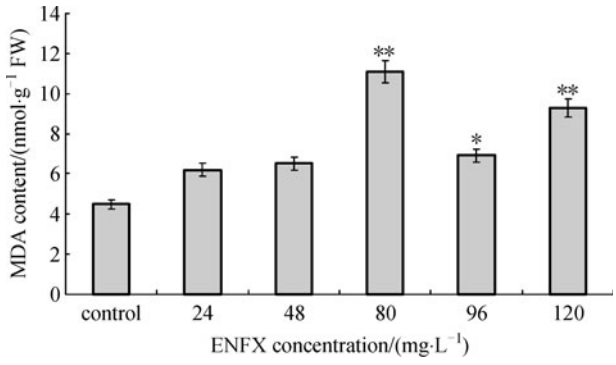

Fig. 6 Effects of different concentrations of ENFX on MDA contents of *S. obliquus* (96 h exposure). *, **: compared with the control group, $p < 0.05$, $p < 0.01$; FW: fresh weight

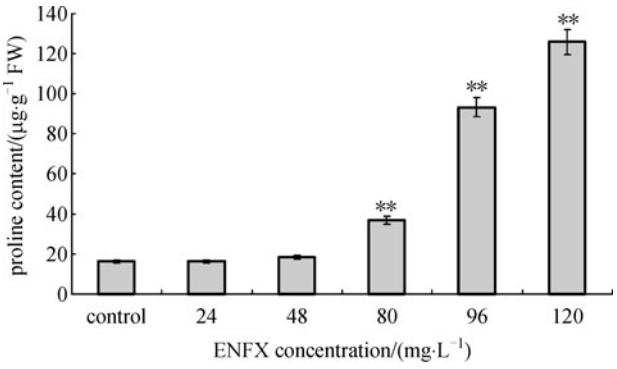

Fig. 7 Effects of different concentrations of ENFX on proline contents of *S. obliquus* (96 h exposure). *, **: compared with the control group, $p < 0.05$, $p < 0.01$; FW: fresh weight

the control was observed under the influence of exogenous 96 and 120 mg·L^{-1} ENFX on the 4th day of culture. This indicated that algae cells underwent serious oxidative damage. However, Altinordulu and Eraslan reported none of enrofloxacin, ciprofloxacin, and norfloxacin at therapeutic doses for three days could cause the generation of an intolerable level of free radicals or subsequent lipid peroxidation in chicks [47]. The difference might be due to the various species used in studies. Also MDA could inhibit the SOD activity [48].

3.3.5 Effects on proline content

It is well known that proline is an imino acid and gets accumulation in a wide variety of organisms ranging from bacteria to higher plants on exposure to any type of environmental stresses whether abiotic or biotic stresses, also proline is considered to be one of the first metabolic responses to stress. And proline accumulation is believed to protect plants including algae against stress injury by maintaining osmoregulation, protecting enzyme denaturation, regulating cytosol acidity, and maintaining the NAD^+/NADH ratio [49,50]. In the present study, the accumulation of proline in ENFX-treated aquatic plants of *S. obliquus* was observed, which might be attributed to the strategies adapted by algae to cope up with ENFX toxicity. As shown in Fig. 7, the proline content increased with the increase of ENFX concentration. The proline contents in lower treatment groups were not significantly different from that in the control. However, the proline content increased sharply when ENFX concentration was all about or over 80 mg·L^{-1} ($p < 0.01$), even in 120 mg·L^{-1} exposure group the content of proline was 7.67 folds of the control group. This might be cause by the change in the path of proline metabolism that when algae was subjected to ENFX, the proline oxidation was blocked and the speed of protein synthesis was slower, leading to the elevation of proline mass fraction. Also the high proline accumulation observed in algae, which might result from a loss of cell homeostasis caused by lipid peroxidation in polyunsaturated fatty acids of biomembranes. Some other earlier reports had unequivocally demonstrated that there existed a direct correlation between lipid peroxidation and proline accumulation [51,52]. In the present study, there seemed to be a correlation between the accumulation of proline and lipid peroxidation, because there was a great correlation between the contents of proline and MDA ($R = 0.959$, $n = 6$). Moreover, Lin and Kao reported the proline accumulation participated in the regulation of root growth of rice seedlings under NaCl conditions and proline level in roots was inversely associated with rice seedling root growth [53].

3.4 Mechanism of effects on *S. obliquus* exposed to ENFX

In this study, after ENFX treatment for 96 h, with the increase of the exposure concentration, the contents of photosynthetic pigments decreased, the soluble protein content and the SOD activity increased and then decreased, the of superoxide anion (O_2^-) increased continually, the contents of MDA and proline changed little at first and then increased rapidly. Exposure to lower concentrations of ENFX (less than 80 mg·L^{-1}), the photosynthetic pigments contents decreased. The change might be due to the chloroplast pigments damage caused by ROS [40], and affected the photosynthesis of algal cell. Although the generation rate of ROS indicated by superoxide anion (O_2^-) increased to some extent in algal cells, in the process of ROS generation and conversion, the algae intracellular antioxidant defense system formed by SOD and other enzymes worked, the SOD activity increased and catalyzed the conversion of O_2^- to O_2 and H_2O_2 to eliminate active oxygen free radicals which could induce lipid peroxidation of membrane and other biomolecule damages [42], so the content of lipid peroxidation product MDA was affected slightly. Also the proline content change showed that lower concentration of ENFX had lower damage to the algal cells. The SOD activity increase might be one of the

Table 3 Correlations of the physiologic biochemical indexes

	C-a	C-b	C-k	protein	SOD	MDA	O_2^-	proline
C-a	1	0.942	0.997	0.106	−0.260	−0.867	−0.913	−0.757
C-b		1	0.931	0.053	−0.255	−0.756	−0.921	−0.686
C-k			1	0.171	−0.225	−0.898	−0.932	−0.803
protein				1	0.729	−0.594	−0.373	−0.679
SOD					1	−0.146	0.091	−0.189
MDA						1	0.907	0.959
O_2^-							1	0.908
proline								1

reasonable explanations about the change of protein content, because SOD is an inner original active protein. As the ENFX exposure concentration increased to 80 $mg \cdot L^{-1}$ and over, the photosynthetic pigments contents in algal cells continued to decline, the O_2^- generation rate, MDA and proline contents increased apparently, but the SOD activity decreased rapidly. All the changes might be due to the excessive concentration of ENFX on *S. obliquus*, which could lead to the serious algal cellular damage, and that has been over the regulation range of algal cells. The ROS continuously increased and exceeded the limit of the antioxidant defense system, and then attacked the membrane structures in algae cells, leading to the membrane lipid peroxidation product MDA content increased. Meanwhile, the osmotic equilibrium of algal cells was broken; subsequently the proline synthesis enzymes were activated, resulting in an increase of the proline content and a further proline accumulation. Also the lipid peroxidation can modify protein structures.

Previous studies showed that the toxicities of organic pollutants to organisms including algae were induced primarily through the peroxidation damages of organisms caused by ROS [54,55]. The correlation of the physiologic and biochemical indexes (Table 3) selected in the present study also confirmed this mechanism. So the main toxicity mechanism of ENFX to *S. obliquus* was that algal cells generated the reactive oxygen species under ENFX stress, and then the ROS induced the membrane lipid peroxidation and other biologic macromolecules damages, also changed the biomembrane permeability furtherly.

4 Conclusions

In this study, the toxic effects of ENFX on *S. obliquus* were studied. From the conducted experiment it can be concluded that 1) ENFX can inhibit the algal growth, the 24, 48, 72, and 96 h EC_{50} were 88.39, 63.86, 45.10, and 59.16 $mg \cdot L^{-1}$, respectively; 2) under different concentrations of ENFX treatment, the photosynthetic pigments in algae decreased, and the changes of chlorophyll contents were more sensitive and happened much earlier than the growth, especially chlorophyll-a, therefore, the chlorophyll-a in *S. obliquus* could serve as a more sensitive biomarker and be used for early warning of ENFX exposure; 3) with the increase of ENFX concentration, the generation rate of superoxide anion increased continuously, whereas the proline content and SOD activity increased first and then declined, when the treatment concentration was greater than or equal to 80 $mg \cdot L^{-1}$, the MDA and proline contents were significantly increased; 4) all the results suggested that ENFX had a certain toxic effects on *S. obliquus*, the main toxicity mechanism of ENFX was that algal cells generated the reactive oxygen species under ENFX stress, and then the ROS induced the membrane lipid peroxidation and other biologic macromolecules damages, also changed the biomembrane permeability furtherly.

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