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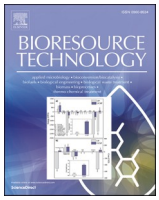

Effects of oxytetracycline and zinc ion on nutrient removal and biomass production via microalgal culturing in anaerobic digester effluent

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HIGHLIGHTS

- Combined stress inhibited more heavily on NH_3-N removal and biomass growth.
- There existed optimal concentrations of Zn(II) and OTC for TP removal.
- Exposure to OTC and 5.0 mg/L of Zn(II) increased TP removal via microalgae.
- High concentrations of Zn(II) and OTC decreased unsaturation degree of fatty acids.
- Nutrient removal mechanisms under stress of OTC and Zn(II) were elucidated.

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ABSTRACT

Removal of nutrients from swine wastewater digester effluent (SWDE) by microalgae *Coelastrella* sp. and production of *Coelastrella* sp. were investigated at the presence of oxytetracycline (OTC) and Zn(II). Mechanisms of stress of OTC and Zn(II) on microalgae were discussed via analyzing the removal performance of SWDE and biochemical characteristics of microalgae. Results showed that removal efficiency of ammonia nitrogen and biomass yield of microalgae at the presence of 5000 μg/L of OTC decreased by 13.1% – 50.1% and 28.2% –71.5%, respectively, when Zn concentration was increased from 0.50 mg/L to 5.0 mg/L. The presence of 5.0 mg/L Zn(II) promoted the accumulation of lipids in microalgae, and the presence of 50 μg/L OTC increased unsaturation of fatty acid methyl ester. Content of glutathione and activity of both glutamine synthetase and superoxide dismutase decreased with the increase of OTC concentration, while content of adenosine triphosphatase increased when Zn(II) concentration was also increased.

1. Introduction

Swine wastewater in which there are high concentrations of ammonia nitrogen (NH_3-N) and total phosphorus (TP) poses challenges for conventional treatment processes (Chen et al., 2020). Compared with other biotreatment in swine wastewater including via bacteria (Wen et al., 2016), duckweeds (Cheng and Stomp, 2009; Zhou et al., 2019), and wetland plants (Klomjek, 2016), microalgal culturing has the advantages of high biomass yield, low secondary pollutant production, good environment adaptability, and high value-added products, so it has great potential for the treatment of and nutrient recovery from swine wastewater digester effluent (SWDE) (Lopez-Pacheco et al., 2021; Nam et al., 2016; Abubackar et al., 2019; Li et al., 2020a). Nutrient metals and antibiotics are often used as an additive in hog feeds to prevent disease and promote growing, which can not be absorbed well by hogs. Consequently, there usually exist antibiotics and nutrient metals in SWDE (Carusso et al., 2018; Li et al., 2020b).

As the most commonly used nutrient metal added to hog feeds, Zn(II)

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is the enzyme cofactor of ribonucleic acid polymerase, superoxide dismutase and carbonic anhydrase in microalgae, which plays an important role in the growth of microalgae (Monteiro et al., 2010). The change of Zn(II) concentration in the growth environment can affect the content of fatty acids and the accumulation of protein and chlorophyll in microalgae (Ajitha et al., 2021). Excessively high concentrations of Zn(II) interfere with the uptake of calcium by microalgae, thus reducing ATPase activity (Liu et al., 2021). Zn(II) can also replace Mg(II) in chlorophyll, thereby altering the composition of chlorophyll and inhibiting photosynthesis. In addition, high concentration of Zn(II) can affect the contents of glutathione (GSH) and malondialdehyde (MDA), and the activity of superoxide dismutase (SOD) in microalgae (Cao et al., 2015; Hamed et al., 2017; Li et al., 2020b). Thus, the biomass production and nutrient removal capacity of microalgae are affected. However, the excessively low concentration of zinc ions in the growth environment cannot meet the needs of rapid growth of microalgae.

There are also some reports about the effect of zinc ion concentration on the growth of microalgae. For example, Li et al., (2020b) investigated the influence of different concentrations of Zn(II) in SWDE on the biomass production and nutrient removal of microalgae *coelastrella* sp., and measured some biochemical characteristics of microalgae, such as the contents of protein, adenosine triphosphate (ATP) and GSH and the activity of SOD. The results indicated that with the increase of Zn(II) concentration, the contents of ATP, protein, GSH, and SOD activity in *coelastrella* sp. all increased. These responses may promote the consumption of TP by *coelastrella* sp. under high Zn(II) concentration. Hamed et al. (2017) also studied the changes of biochemical indexes of *Chlorella sorokiniana* exposed to different concentrations of Zn(II) and reported that low concentration of Zn (II) promoted the growth of *Chlorella sorokiniana*, but it was seriously inhibited at high concentration, and *Chlorella sorokiniana* accumulated a large amount of GSH under Zn (II) stress and showed higher SOD enzyme activity. Ajitha et al. (2021) has indicated that the presence of low concentration Zn (II) promoted the protein accumulation and SOD activity of *Chlorella vulgaris*, while at high concentration, the protein content and SOD activity decreased with the increase of Zn(II) concentration. The above studies demonstrated that the change of Zn(II) would affect the growth and biochemical characteristics of microalgae, thus affecting the biomass output and nutrient removal of microalgae.

On the other hand, antibiotics in SWDE may also affect the biomass output and nutrient removal capacity of microalgae. Oxytetracycline (OTC) is an effective and low-cost antibiotic that widely used in hog feeds, but most of the OTC added to feed is difficult to be absorbed and metabolized by the digestive tract of pigs, and discharged from the kidney and biliary tract in its original chemical form (Ferreira et al., 2007). Therefore, high concentrations of OTC in SWDE are often discharged into natural water bodies. Although these OTC are easily photodegradable in the light transmission zone of water body, most OTC still exist in the biological retention tank of SWDE, so it may exist in sediments for a long time and have a serious impact on water organisms, especially microalgae (Siedlewicz et al., 2020). OTC can bind to the A position in the 30 S of ribosomes in water biological cells, replacing the binding of aminoacyl-TrNA at this position, thus inhibiting the synthesis of peptide chains, ultimately affecting the synthesis of some proteins and inhibiting biological growth (Hu et al., 2021).

Some studies on the effect of OTC concentration on the growth of microalgae have been reported. For example, Siedlewicz et al. (2020) have reported the influence of a wide range concentration of OTC on Chlorella vulgaris growth, chlorophyll *a*, the optical density and cell number were analyzed, results showed that OTC could disrupt process of photosynthesis via decrease PS II efficiency in microalgae. It was also reported that OTC promoted the growth of microalgae at low concentration and inhibited the growth at high concentration, moreover, with the increase of OTC concentration, the protein content and SOD enzyme activity of microalgae increased significantly (Zhou et al., 2020). These studies indicate that OTC may have unexpected effects on biomass production and nutrient removal of microalgae when microalgae are used to treat SWDE. Most importantly, the carbonyl and hydroxyl groups in OTC and its derivatives can chelate with variety of metal cations, including Cu(II), Pb(II), Cd(II), Ca(II) and Mg(II) (Werner et al., 2006; Zhao et al., 2013), which may also chelate with Zn(II) and affect the growth of microalgae.

Previous studies have investigated the effect of either antibiotics or Zn(II) on microalgae, and mechanisms have been revealed via the analysis of SOD, GSH and others enzymes (Mao et al., 2021, Li et al., 2020b). However, there are few reports on the combined effects of OTC and zinc ion on biomass production of microalgae and nutrient removal from SWDE, and such reports are very meaningful due to the simultaneous existence of OTC and zinc ion in SWDE. It will be meaningful if there exists an optimal concentration of both Zn(II) and OTC for microalgal growth and SWDE treatment. It is also pretty intrigued if the mechanisms of the combined effects could be elucidated via the analysis of proteins, SOD, GSH, Glutamine synthetase and ATP in microalgae. These results could lead to a better understanding of the performance of microalga-based systems for SWDE treatment, and consequently a better design and operation of such systems.

2. Materials and methods

2.1. Microalgal strain

The microalgal strain was collected by Luo et al. (2018), who isolated these microalgae from a pond near the piggery farm in Hunan, China (26°36′95″N, 112°05′08″E), and dominant unit strain was cultivated in BG 11 with sterile distilled water. Homology of this microaglae showed 97% similar to *Coelastrella* sp., and the primitive microalgal strains were deposited in Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), Wuhan, China, and strain number was FACHB-2400.

2.2. Swine wastewater digester effluent

Swine wastewater digester effluent (SWDE) was collected from a local piggery farm (27°24′03″N, 113°07′06″E), and the hogs were breed without any feed from birth, it is guaranteed there free of heavy metals and antibiotics in their excreta. Supernatant of SWDE was collected and removed suspended solid via sedimentation (static settlement for 2 h) and filtration (suction filtration for 3 times). The characteristics of SWDE in this study were showed in Table 1.

2.3. Chemicals

$ZnSO_4$ was used as the source of Zn(II) (Analytically Pure, Sinopharm Chemical Reagent Co. LTD, China), Oxytetracycline hydrochloride (High Purity Grade, Bomei Biotechnology Co., LTD, Hefei, China) was used as the source of Oxytetracycline (OTC).

2.4. Experimental procedures

Two constant concentration levels of Zn(II) of 0.5 mg/L and 5.0 mg/L and three concentration levels of OTC including 50, 500 and 5000 μg/L were studied. In this study, *Coelatrella* sp. was inoculated to the 20%

Table 1
Physicochemical characteristics of SWDE.

Parameter	Unit	Concentration
pH	–	8.3 ± 0.4
NH_3-N	mg/L	88 ± 10
TP	mg/L	9 ± 1
OTCs	μg/L	Undetected
Zinc ions	μg/L	4.2 ± 0. 5

pretreated SWDE (diluted with distilled water) with various concentrations of Zn(II) and OTC. Microalgae were cultured in 500 mL conical flasks (400 mL 20% pretreated SWDE), biomass concentration was controlled at OD_{680} 0.10 after inoculation, kept microalgae in illuminating incubator with 100 ± 10 μmol m^{-2} s^{-1} light intensity of fluorescent lights, and 25 ± 1 °C temperature, daily / dark was 12:12 h, conical flasks were shook three times each day, culture time was 10 days. Zn(II) and OTC concentration were measured and supplemented every 12 h to maintaining a stable concentration level.

2.5. Chemical analysis

Zinc concentration in SWDE was analyzed by atomic absorption spectroscopy (AAS, PEAA700, Waltham, Massachusetts, U.S.A.). Concentration of OTC was according to the method described by Lin et al. (Lin et al., 2019) and analyzed using high performance liquid chromatography (HPLC, Angilent, Palo alto, California, U.S.A.) equipped with Kromasil C18 column (5 um × 4.6 mm × 250 mm) and UV detection. SWDE was collected by centrifugation, and supernatant was percolate trough 0.22 μm cellulose acetate filter and analyzed via HPLC. HPLC parameter of OTC: detector wavelength 352 nm; mobile phase 0.01 M formic acid : actonitrile = 80 : 20; flow rate 0.2 mL/min; injection volume 20 μL and column temperature at 30 °C.

Concentrations of NH_3-N and TP were measured via Nessler's reagent colorimetric methods and potassium persulfate digestion methods (Zhou et al., 2018), respectively.

Biomass of *Coelastrella* sp. was calculated using a modified method by Li et al. (2018), dry weight was measured as follow Eq:

$$\text{Dry Weight (g/L)} = 0.3357 \times OD_{680},\ R^2 = 0.9962 \quad (1)$$

Lipids were extracted from dry biomass: weighing 0.1 g dry biomass of microalgae into a 15 mL screw–top glass tube, and adding 10 mL of methanol-chloroform (v/v = 1 : 2) mixture. Tube was sonicated at maximum intensity for 1 h, and extracted overnight in shaker with 130 rpm and 27℃. After 24 h, extract was percolate trough 0.22 μm nylon syringe filter to remove the algal solid residues. Filtrate was transferred to pre-weighed screw-top glass tube, and 2 mL water was added in tube for shaking 1 min, and then waiting 30 min for liquid separate. After that, the upper aqueous phase was removed carefully, and tube was evaporated in oven at 50 ℃ to dryness. The weight change of tube divides the dry biomass is the content of lipid. Total lipids were measured via gravimetric analysis, and the content of lipids in microalgae was expressed as lipid/dry weigh × %.

Fatty acid methyl esters (FAMEs) were prepared with an extraction transesterification according to Song et al. (2013) and the composition determine was analyzed by Gas Chromatography-Mass Spectrometry (GC–MS, QP2010, Shimadzu, Japan). 0.1 g dry biomass of microalgae was added in 25 mL screw–top glass tube, which contains 10 mL fresh solutions of a mixture of chloroform, contrated sulfuric acid and methanol (5 : 0.75 : 4.25). The tube was carried out in shaking bath for 90 min at 90℃, next the tube was cooled to room tempertature, and then adding 2.5 mL water and shake 1 min for mixture. After phases separated, the upper aqueous phase was removed carefully, the lower phase was transferred to a 10 mL tube with anhydrous Na_2SO_4 for dehydrate. The dehydrated sample was percolate trough 0.22 μm nylon syringe filter to remove the Na_2SO_4, and analyzed by GC–MS. NIST Mass Spectral Database and quantified by the area normalization method were used to identified the compounds of FAME.

The unstauration degree (UD) was calculated according to Redel-Macías et al. (2013) as follows:

$$UD = (3\times\%_T + 2\times\%_D + 1\times\%_M) / 100 \quad (2)$$

where $\%_T$, $\%_D$ and $\%_M$ are the percentages in weight of tri-unsaturated, di-unsaturated and mono-unsaturated methyl esters, respectively.

2.6. Measurements of microalgal biochemical properties

Physiological and biochemical of microalgae was measured in day 4, when biomass growth was the fastest. The content of proteins and adenosine triphosphate (ATP), glutathione (GSH), the activity of glutamine synthetase (GS) and superoxide dismutase (SOD) were measured using assay kits (Shenzhen Ziker Biological Technology Company, Shenzhen, China). The content of chlorophyll *a* (Chl *a*) in microalgal biomass was measured according to the method deriving by Li et al. (2016).

2.7. Statistical analysis

All experiments were performed in triplicate, and the mean ± SD (standard deviation) were showed for describe results.

3. Results and discussion

3.1. Effect of OTC on microalgal nutrient removal at constant zinc concentrations

3.1.1. Dynamic changes of concentrations of OTC and zinc

Previous studies have reported that OTC can chelate with metal cations, such as Cu(II), Pb(II), Cd(II), Ca(II) and Mg(II), thus this study investigated whether Zn(II) can be chelated with OTC. Fig. 1 showed the Dynamic changes of concentrations of OTC and Zinc in 12 h.

As showed in Fig. 1a, concentration of OTC decreased rapidly in all groups within 2 h, because microalgae could adsorb OTC when exposed in SWDE (Choi et al., 2020). In the first 2 h, concentration of OTC decreased to 62.0%, 56.0% and 66.0% on the group of 50 μg/L OTC, 50 μg/L OTC + 0.50 mg/L Zn(II) and 50 μg/L OTC + 5.0 mg/L Zn(II), respectively. After 2 h, concentration of OTC continues to decreased at all experience groups, and the lowest concentration of OTC was occurred in the presence of 5.0 mg/L Zn(II). Fig. 1b and Fig. 1c showed the same results, concentration of OTC decreased rapidly in the first 2 h and has more decreased in the presence of 5.0 mg/L Zn(II).

In order to illustrate the change of OTC concentration by Zn(II) concentration, this study further measured the change of Zn(II) in different experimental groups. As showed in Fig. 1d, concentration of Zn (II) was decreased within 2 h in all groups, which was corresponds to the change of OTC concentration in Fig. 1a, Fig. 1b and Fig. 1c, and concentration of Zn(II) was decreased with concentration of OTC increased. It is exciting that the lowest concentration of Zn(II) was occurred in the presence of 5000 mg/L OTC, which means high concentration of OTC and Zn(II) could mutual promote the concentration decreased. Results showed that Zn(II) could chelate with OTC, especially in presence of high concentration of Zn(II) and OTC, which might influence microalgae in SWDE treatment.

3.1.2. Effect of OTC on microalgal NH_3-N removal at constant zinc concentrations

Different concentrations of OTC were introduced into SWDE with two constant Zn(II) concentration, and their effects on the removal of ammonia nitrogen and total phosphorus by microalgae *Coelatrella* sp. were shown in Fig. 2. It can be observed that compared with the blank group without any stress (71.3%), when only 0.50 mg/L Zn(II) was present in SWDE, the removal rate of NH_3-N by *Coelatrella* sp. was about 73.0% on the 10th day, which was similar to the experimental results reported in previous studies (Li et al., 2020b).

Therefore, further research was carried out on this basis, Zn(II) was kept at at 0.50 mg/L, and different concentrations of OTC were added to culture *Coelatrella* sp. It can be found that when 50 μg/L OTC was added, the NH_3-N removal efficiency by *Coelatrella* sp. was about 73.3%, but with the increased of OTC concentration, the NH_3-N removal efficiency gradually decreased. In the presence of 500 and 5000 μg/L OTC, the NH_3-N removal efficiency was reduced to 68.0% and 55.9%,

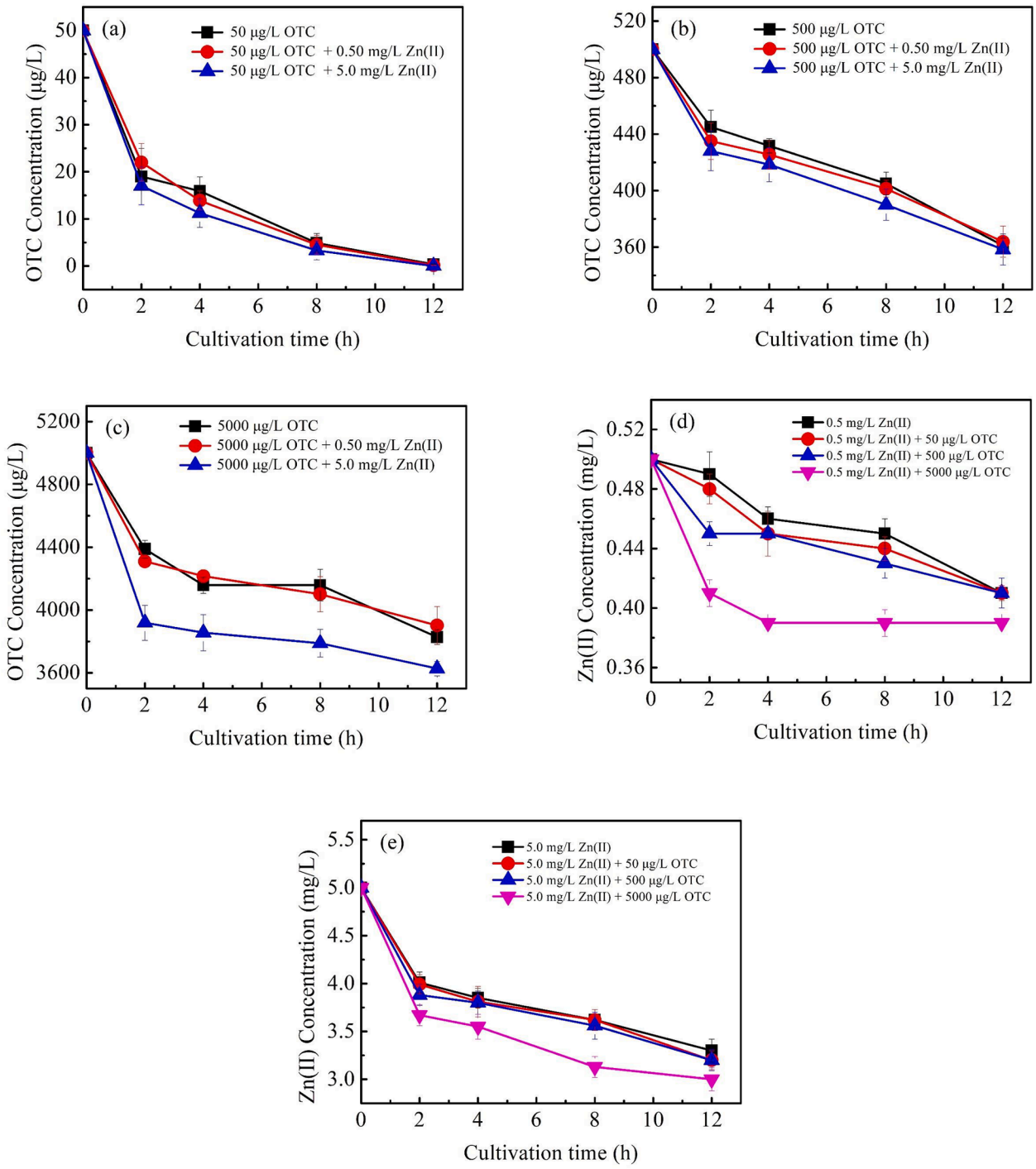

Fig.1. Dynamic changes of concentrations of OTC (a,b,c) and Zn(II) (d,e) at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in *Coelastrella* sp. treating SWDE.

respectively.

As shown in Fig. 2c, when there was only 5.0 mg/L Zn(II) in SWDE, it would inhibit the removal of NH_3-N by *Coelatrella* sp., and the removal efficiency of NH_3-N was about 68.7%. Interestingly, under combined stress of 5.0 m/L Zn(II) + 50 μg/L OTC and 5.0 mg/L Zn (II) + 500 μg/L OTC, the removal efficiency of NH_3-N were 68.5% and 68.7% respectively, which was almost no difference from that under Zn (II) stress alone. However, in the experimental group of 5.0 mg/L Zn (II) + 5000 μg/L OTC, the removal efficiency of NH_3-N decreased to 18.9%. The results indicated that in the combined stress of Zn (II) and OTC, the concentration of Zn (II) mainly affects the removal efficiency of NH_3-N, and OTC affected the NH_3-N removal by *Coelatrella* sp. only at a higher concentration.

The effect of different concentrations of OTC on the removal of NH_3-N by *Coelatrella* sp. without Zn (II) stress as shown in Fig. 2a. The results indicated that the removal efficiency of NH_3-N from SWDE by *Coelatrella* sp. did not change in the presence of low concentration OTC. On the 10th day of culture, in the experimental group added with 50 μg/L OTC and 500 μg/L OTC, the removal efficiency of NH_3-N reached 71.3% and 71.2%, respectively. However, in the experimental group with 5000 μg/L OTC, the removal efficiency of NH_3-N decreased to 69.0%. This was because the Zn(II) in SWDE may stimulate or inhibit chlorophyll,

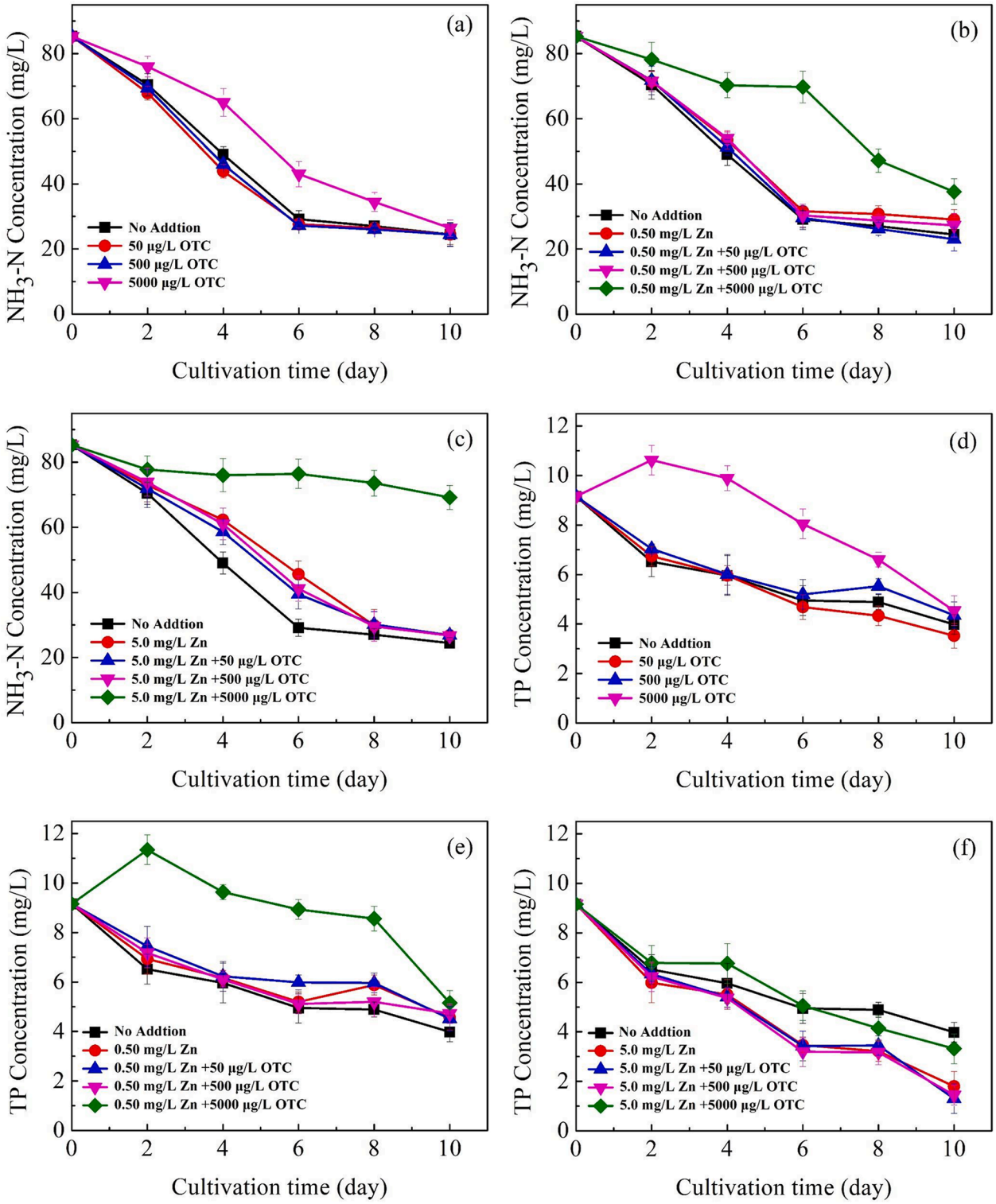

Fig.2. Dynamic changes of concentrations of NH_3-N (a,b,c) and TP (d,e,f) at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in *Coelastrella* sp. treating SWDE.

glutathione and glutamine synthetase in *Coelatrella* sp., which determine the absorption of NH_3-N and intracellular transformation of *Coelatrella* sp., and affect the biomass production of *Coelatrella* sp. cells. (Buayam et al., 2019; Dharmawardene et al., 1973;)

3.1.3. Effect of OTC on microalgal TP removal at constant zinc concentrations

As shown in Fig. 2e, the presence of 0.5 mg/L Zn(II) and 0.5 mg/L Zn (II) combined with different concentrations of OTC would reduce the removal efficiency of TP in SWDE by microalgae *Coelatrella* sp., and the TP removal efficiency decreased with the increase of OTC concentration. On the 10th day of culture, the TP removal efficiency in experimental

groups of 0.50 mg/L Zn(II), 0.5 mg/L Zn(II) + 50 μg/L OTC, 0.5 mg/L Zn (II) + 500 μg/L OTC, and 0.5 mg/L Zn(II) + 5000 μg/L OTC were 50.2%, 50.7%, 48.4% and 43.7%, respectively. Especially in the experimental group added with 0.5 mg/L Zn(II) and 5000 μg/L OTC, the presence of OTC would significantly inhibit the removal of TP by *Coelatrella* sp., and the content of TP in SWDE did not decrease, instead increased in the first two days of the experiment. This may be because when *Coelatrella* sp. encounter high concentration of acute stress, algal cells die and rupture, thus releasing intracellular substances including phosphorus (Ajitha et al., 2021).

As shown in Fig. 2f, the presence of 5.0 mg/L Zn (II) alone promoted the removal of TP by microalgae *Coelatrella* sp., and the TP removal efficiency reached 80.4% on the 10th day of culture. Compared with the condition of Zn (II) alone, the presence of 5.0 mg/L Zn(II) + 50 μg/L and 5.0 mg/L Zn(II) + 500 μg/L can slightly promote the removal of TP by *Coelatrella* sp., and the TP removal efficiency reached 85.8% and 84.2% on the 10th day, respectively. Compared with the condition of Zn (II) alone, 5.0 mg/L Zn (II) + 5000 μg/L significantly inhibited the removal of TP by *Coelatrella* sp., but compared with the blank group without any stress, it still promoted the removal of TP by *Coelatrella* sp. after 6 days of culture, and the removal efficiency was about 63.7%. The experimental results showed that in the combined stress of Zn (II) and OTC, the main factor affecting the removal of TP was also the concentration of Zn (II). Only at a higher concentration, OTC could affect the removal of TP by *Coelatrella* sp. Fig. 2d showed the removal of TP by *Coelatrella* sp. with different concentrations of OTC without Zn (II). The results indicated that the effect of OTC on the removal efficiency of TP by microalgae was not obvious at low concentrations, especially in the first 4 days of culture. However, from the 4th day to the 10th day of culture, the TP removal efficiency decreased with the increase of OTC concentration. On the 10th day of culture, the TP removal efficiency was about 61.5% in the experimental group containing 50 μg/L of OTC, and 52.5% and 50.4% in the experimental group containing 500 μg/L and 5000 μg/L of OTC, respectively.

In SWDE, the reduction of TP is mainly due to biological assimilation and sedimentation, in which about 3–23% of phosphorus can be assimilated and utilized by microalgae (Abou-Shanab et al., 2013). At the same time, microalgae need to synthesize corresponding proteins and enzymes to prevent cell oxidative death under environmental stress, which may stimulate the consumption of phosphorus. Therefore, the removal efficiency of TP in SWDE increases with the increase of phosphorus consumption by microalgae (Li et al., 2020b).

The above results indicated that when using the microalgae *Coelatrella* sp. to remove nutrients in SWDE which contained Zn(II) , low concentration of OTC (50–500 μg/L) have little effect on the removal of NH_3-N and TP by *Coelatrella* sp., and even promote a small amount, but the presence of high concentration OTC (5000 μg/L) will reduce the removal of NH_3-N and TP by microalgae.

3.2. Effect of OTC on microalgal growth at constant zinc concentrations

Fig. 3 showed the biomass production of *Coelatrella* sp. in SWDE under two fixed concentrations of Zn (II) and different concentrations of OTC stress. As shown in Fig. 3a, in the experimental group without Zn (II), low concentration OTC (50–500 μg/L) had no significant effect on the growth of *Coelatrella* sp. On the 10th day of culture, the microalgae biomass in the experimental group with 50 μg/L and 500 μg/L of OTC increased slightly compared with the experimental group without addition, and the biomass was 0.316 and 0.312 g/L, respectively. However, in the experimental group containing 5000 μg/L of OTC, microalgae biomass was significantly inhibited, which was about 0.282 g/L on the 10th day, this result was similar with Bashir and Cho (2016), who has studied the effect of tetracycline on two microalagae. Under OTC stress, the change law of *Coelatrella* sp. biomass was related to the removal efficiency of NH_3-N and TP.

As shown in Fig. 3b, under the fixed concentration of 0.5 mg/L Zn (II)

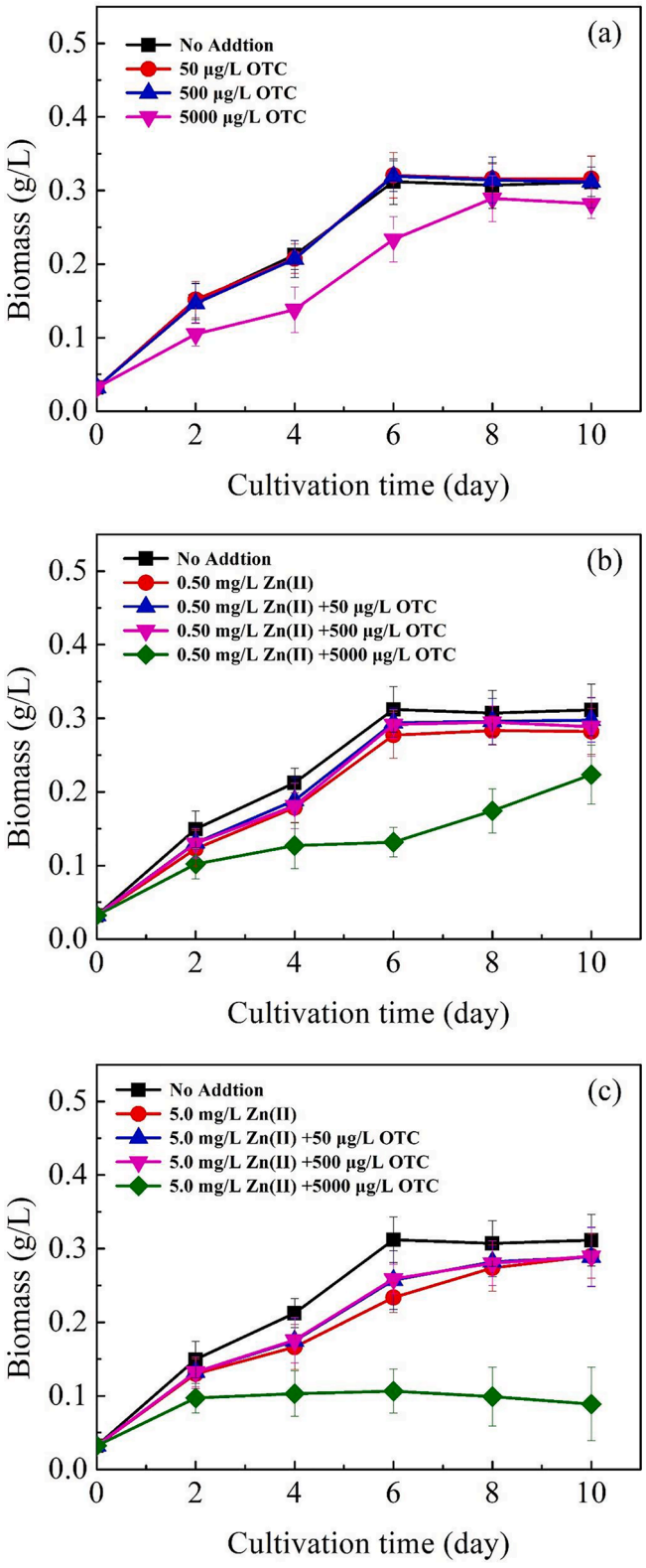

Fig.3. Dynamic changes of biomass of *Coelastrella* sp. be cultured at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in SWDE.

and its combined stress with different concentrations of OTC, the growth of *Coelatrella* sp. was inhibited compared with the experimental group without additive stress, especially at 0.5 mg/L Zn (II) + 5000 μg/L, there was a significant inhibition (decreased to 0.223 g/L), and the inhibition

efficiency was about 28.2% on the 10th day of the experiment. However, compared with the single Zn (II) stress group, the biomass output of *Coelatrella* sp. in the experimental group with 50 and 500 μg/L OTC was higher. A similar phenomenon also occurred in the experimental group with a fixed concentration of 5.0 mg/L Zn(II). As shown in Fig. 3c, the biomass output of *Coelatrella* sp. in the experimental group with 5.0 mg/L Zn(II) alone decreased significantly compared with the experimental group without additive stress. Especially in the experimental group with 5.0 mg/L Zn (II) + 5000 μg/L OTC, the biomass of *Coelatrella* sp. decreased by 71.5% (decreased to 0.089 g/L). The experimental results indicated that when Zn(II) was present in SWDE, low concentration OTC (50–500 μg/L) can alleviate the inhibitory effect of Zn(II) on *Coelatrella* sp. growth, which may be because on the one hand, low concentration OTC and their derivatives can provide a certain nutrient source for *Coelatrella* sp. and promote its growth, on the other hand, OTC can form chelates with Zn(II), thus reducing the toxicity of Zn (II) to *Coelatrella* sp. cells. Combined with the results in section 3.1.2, it can be seen that NH_3-N removal efficiency showed highly correlated with biomass production in microalgae treated SWDE. This result was in accordance with Li et al. (2020b), who reported microalgae stress to various concentration of Zn (II) in swine wastewater treatment. It means increasing the biomass of microalgae was an effective way to improve the removal of NH3-N, even under different cultivate conditions.

3.3. Effects of OTC on lipids content and FAME composition in microalgae at constant zinc concentrations

The changes of cell lipids content and fatty acid methyl ester composition of *Coelastrella* sp. after SWDE treatment under the combined stress of two fixed concentrations of Zn (II) and different concentrations of OTC were shown in Fig. 4. The lipids content in *Coelastrella* sp. per gram of dry weight is expressed in g/g. The results indicated that when there was only 0.50 mg/L Zn (II) in SWDE, the lipids content in *Coelastrella* sp. was reduced by 14.2% to about 0.115 g/g compared with the experimental group without additive stress. With the increase of the concentration of introduced OTC, the lipids content in *Coelastrella* sp. increased to 0.102, 0.120 and 0.123 g/g in the experimental group containing 0.5 mg/L Zn(II) + 50 μg/L OTC, 0.5 mg/L Zn (II) + 500 μg/L OTC and 0.5 mg/L Zn(II) + 5000 μg/L OTC, respectively. The results showed that the presence of OTC could promote the accumulation of lipids in *Coelastrella* sp., and the promoting effect increased with the increase of OTC concentration, which was similar to the results reported by Xie et al. (2019). Similar results were found in the experimental group under combined stress of 5.0 mg/L Zn(II) and different concentrations of OTC. As shown in Fig. 4b, when only 5.0 mg/L Zn (II) was present in SWDE, the lipids content in *Coelastrella* sp. increased to 0.137 g/g compared with the experimental group without addition and the experimental group with 0.5 mg/L Zn(II). Different from the

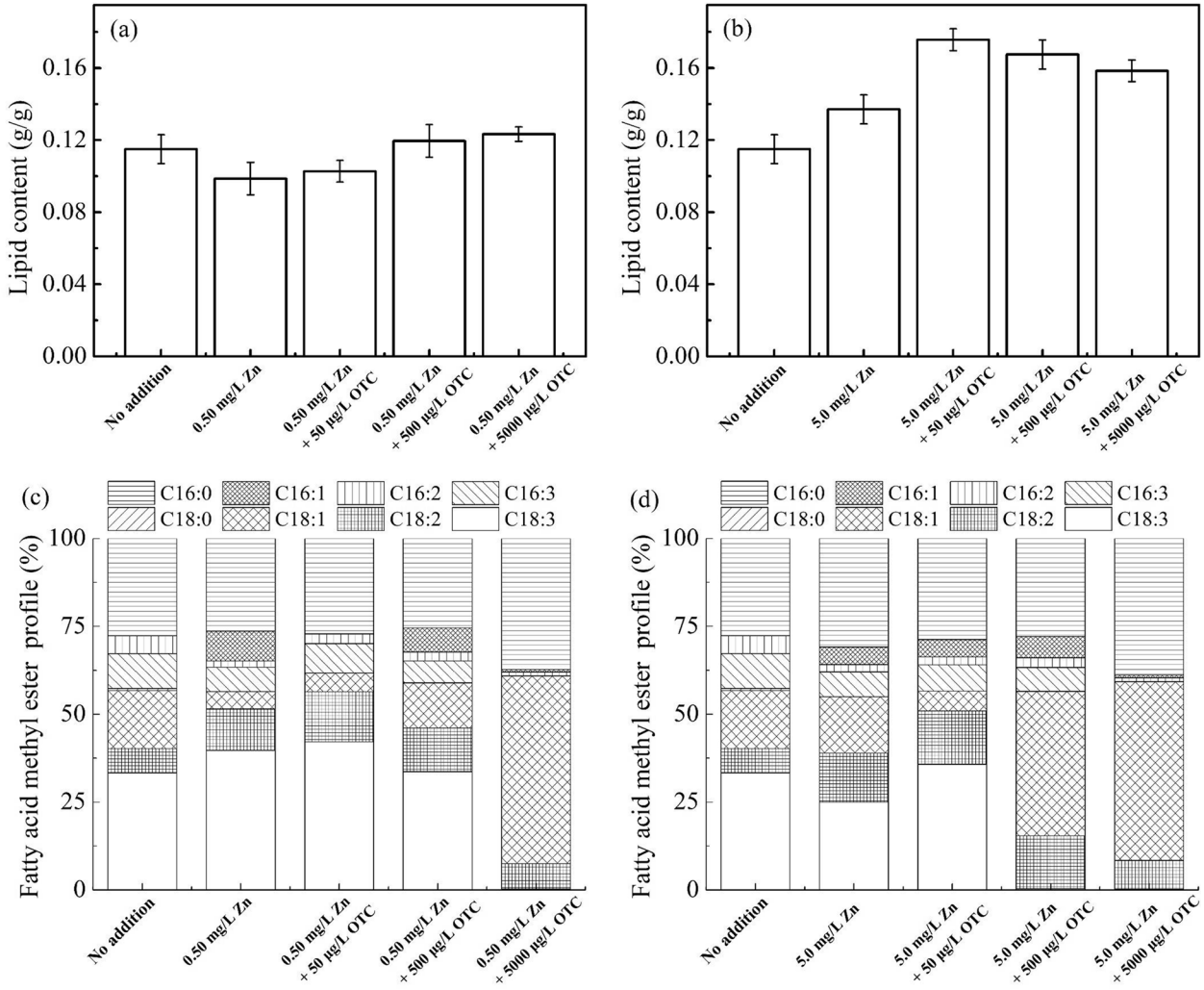

Fig.4. Lipid content (a,b) and FAME compositions (c,d) for *Coelastrella* sp. be cultivated at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in SWDE on day 10. (C16:0, Hexadecanoic acid; 16:1, Hexadecenoic acid; 16:2, Hexadecadienoic acid; 16:3, Hexadecatrienoic acid; 18:1, Oleic acid; 18:2, linoleic acid; 18:3, linolenic acid).

compound stress of 0.50 mg/L Zn (II), in the compound stress of 5.0 mg/L Zn (II) and OTC, although OTC still promoted the accumulation of lipids in *Coelastrella* sp., the increase of lipids content decreased with the increase of OTC concentration. In the experimental group containing 5.0 mg/L Zn(II) + 50 μg/L OTC, the lipids content reached the highest value of 0.176 g/g. With the increase of OTC concentration to 500 and 5000 μg/L, the lipids content decreased to 0.167 and 0.158 g/g, respectively. The experimental results showed that the presence of OTC and 5.0 mg/L Zn (II) could promote the accumulation of liquids in *Coelastrella* sp., because the environmental pressure can play a positive role in the accumulation of lipids in microalgae. When *Coelastrella* sp. are stressed, the reactive oxygen species in cells are unbalanced, which further leads to the oxidative stress response of increased lipids accumulation, and has the greatest promoting effect under the combined stress with low concentration of OTC and high concentration of Zn(II) (Poh et al., 2020).

Combined with the results in section 3.2, it can be find that concentration of OTC at 50–500 μg/L caused slight effect to microalgal biomass and lipid product, even if composite with 0.50 mg/L Zn(II). Only the composite concentration of Zn(II) increased to 5.0 mg/L or OTC increased to 5000 μg/L, the biomass and lipid production showed obvious change.

The properties of fatty acid methyl esters (FAME) are often used to indicate the quality and potential of biodiesel. The major fatty acids accumulation in microalgae were C14:0, C16:0, C16:1, C18:1 and C18:3 (Lopez-Pacheco et al., 2021), C16 and C18 series FAME are used to evaluate the quality and lipids production efficiency of *Coelastrella* sp. biodiesel, the higher the unsaturation of FAME, the lower the kinematic viscosity, pour point and melting point of biodiesel (Luo et al., 2016).

Fig. 4c showed the changes of FAME in *Coelastrella* sp. under the combined stress of different concentrations of OTC and two fixed concentrations of Zn(II) in SWDE. The C16 and C18 fatty acids mainly contained in *Coelastrella* sp. are linolenic acid (18:3), linoleic acid (18:2), Oleic acid (18:1), Hexadecatrienoic acid (16:3), Hexadecadienoic acid (16:2), Hexadecenoic acid (16:1) and Hexadecanoic acid (16:0). As shown in Fig. 4c, compared with the experimental group without addition, a single 0.5 mg/L Zn (II) stress significantly increased the ratio of C18:3 and C18:2 in the FAME of *Coelastrella* sp., and the unsaturation increased from 1.7 to 1.8. With the introduction of OTC, in the experimental group of 0.5 mg/L Zn(II) + 50 μg/L OTC, the ratio of C18:3 to C18:2 further increased. In addition, compared with the single stress of 0.5 mg/L Zn (II), the ratio of C16:3 to C16:2 also increased slightly, and the unsaturation further increased to 1.9. However, with the increase of OTC concentration, the content of polyunsaturated fatty acids began to decrease in the experimental group of 0.5 mg/L Zn(II) + 500 μg/L OTC, and the unit unsaturated fatty acids C18:1 and C16:1 increased, so the unsaturation decreased to 1.7, especially at in the experimental group of 0.5 mg/L Zn(II) + 5000 μg/L OTC, the unsaturation even decreased to 0.7.

Similar results were also observed in the experimental group with a fixed concentration of 5.0 mg/L Zn(II), as shown in Fig. 4d. Compared with the experimental group without addition, in the experimental group under single Zn (II) stress, the C18:2, C18:1 and C16:1 in the FAME of *Coelastrella* sp. increased, C18:3 and C16:3 decreased, and the unsaturation decreased to 1.49. With the introduction of OTC, the unsaturation increased to 1.9 in the experimental group added with 5.0 mg/L Zn(II) + 50 μg/L OTC. However, with the increase of OTC concentration, the unsaturation in experimental groups of 5.0 mg/L Zn(II) + 500 μg/L OTC and 5.0 mg/L Zn(II) + 5000 μg/L OTC decreased to 1.0 and 0.7 respectively, which was far lower than that in the experimental group with the same concentration of OTC combined with 0.50 mg/L Zn (II). The experimental results indicated that Zn (II) and OTC at low concentration can promote the accumulation of unsaturated fatty acids in *Coelastrella* sp. and reduce the proportion of saturated fatty acids.

3.4. Effects of OTC on chlorophyll a and protein content in microalgae at constant zinc concentrations

Compared with the response to OTC, microalgae are more sensitive to the change of Zn (II). Previous studies have described the impact mechanism of Zn (II) on microalgae (Li et al., 2020b), but the impact mechanism of Zn (II) and OTC combined stress on microalgae treatment of SWDE is not clear, so this study investigate it by measuring a series of biochemical indexes of microalgae.

Chlorophyll *a* (Chl *a*) content is often used to evaluate the photosynthetic rate and total biomass of microalgae, as well as the short-term inorganic chemical toxicity of microalgae (Perez et al., 2006). At the same time, harsh environment will induce microalgae to produce ·OH, H_2O_2 and $\cdot O_2^-$, thus affecting the synthesis of Chl *a*. The Chl *a* content per gram of *Coelatrella* sp. is expressed in mg/g. As shown in Fig. 5a, the Chl *a* content of *Coelatrella* sp. in the experimental group of 0.5 mg/L Zn (II) was 2.7 mg/g, which increased slightly compared with the experimental group without addition. When OTC with a concentration of 50–500 μg/L were introduced, there was no obvious effect on Chl *a* content, but the Chl *a* content was significantly reduced to 2.11 mg/g in the experimental group of 0.5 mg/L Zn (II) + 5000 μg/L OTC. This may be because the enzymes related to Chl *a* synthesis in *Coelatrella* sp. are not sensitive to OTC stress, and it will be affected only in the presence of high concentration OTC.

As shown in Fig. 5b, compared with the experimental group without addition, the Chl *a* content of *Coelatrella* sp. decreased from 2.56 to 1.94 mg/g under a single stress of 5.0 mg/L Zn (II). The Chl *a* content decreased to 2.09 and 2.0 mg/g in the presence of 5.0 mg/L Zn(II) + 50 μg/L OTC and 5.0 mg/L Zn(II) + 500 μg/L OTC respectively, reaching the lowest value of 1.56 mg/g in experimental group of 5.0 mg/L Zn(II) + 5000 μg/L OTC. The results showed that the enzymes related to Chl *a* synthesis were more sensitive to the stress of 5.0 mg/L Zn (II). Excessive Zn (II) in SWDE could replace Mg (II) and Fe (II) in protein sulfhydryl (-SH) in *Coelatrella* sp. to synthesize Zn-SH. Moreover, excessive OTC enhanced the oxidation effect of *Coelatrella* sp. and interfered with the integrity and catalysis of enzymes related to photosynthetic pigment biosynthesis (Siedlewicz et al., 2020) Therefore, it inhibited the synthesis of Chl *a* and ultimately affected the growth and nutrient removal of *Coelatrella* sp.

Protein is an important index to evaluate the degree of microalgae under external stress. In this study, it is used to indirectly reflect the enzyme level of *Coelatrella* sp. under stress. In this experiment, g/g was used to express the protein content per gram of *Coelatrella* sp. As shown in Fig. 5c, compared with the experimental group without addition (0.18 g/g), the protein content of *Coelatrella* sp. in the experimental group with 0.5 mg/L Zn(II) increased significantly (0.28 g/g), and further increased with the introduction of 500–5000 μg/L OTC. Similar results also appeared in the experimental group of 5.0 mg/L Zn (II) combined with OTC. As shown in Fig. 5d, the protein content increased to 0.35 g/g in the experimental group of 5.0 mg/L Zn (II), and the introduction of 5000 μg/L OTC further increased the protein content to 0.60 g/g. These results were similar to the previous reports of Ajitha et al., (2021) and Sabatini et al., (2009).

3.5. Effects of OTC on SOD activity and glutathione content in microalgae at constant zinc concentrations

The experimental results indicated that when Zn (II) and OTC exist in the growth environment, microalgae will synthesize a series of protective proteins and enzymes to prevent cell oxidation (Sabatini et al., 2009), including plant chelating peptidase (Grill et al., 1988) capable of synthesizing glutathione (GSH) and antioxidant superoxide dismutase (SOD). In order to further elaborate the oxidative stress response of microalgae under the combined stress of Zn (II) and OTC, GSH and SOD were analyzed in this study.

The role of SOD in microalgae is mainly to prevent the free radicals

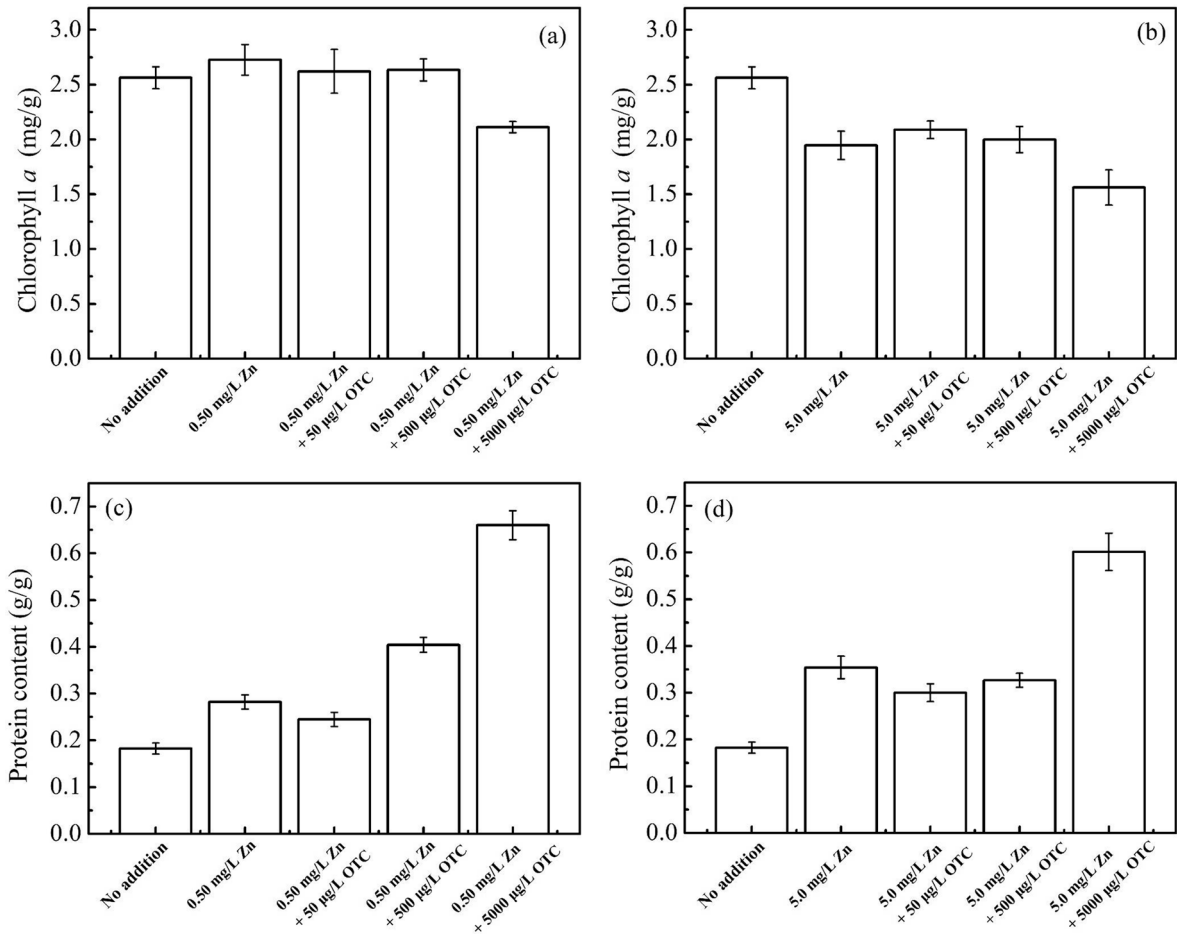

Fig.5. Content of chlorophyll *a* (a,b) and protein (c,d) of *Coelastrella* sp. at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in SWDE on day 4.

produced by cell metabolism from damaging cells. It can reduce the concentration of hydrogen peroxide by clarifying superoxide free radicals. If SOD defense fails, excessive reactive oxygen species will lead to damage including lipid peroxidation, thereby inhibiting microalgae growth and nutrient removal (Aderemi et al., 2018). The effect of combined stress of Zn (II) and OTC on SOD activity in *Coelastrella* sp. was shown in Fig. 6a, Fig. 6b and Table 2. The SOD activity contained in each gram of *Coelastrella* sp. was expressed in U/g. The presence of single OTC can promote SOD activity, and the SOD activity decreased with the increase of OTC concentration. A single 0.50 mg/L Zn (II) has no obvious effect on the SOD activity of *Coelastrella* sp., but the SOD activity increased significantly after the introduction of OTC. The results showed that low concentration of Zn (II) and OTC combined stress promoted the SOD activity of *Coelastrella* sp., and the SOD activity decreased with the increase of OTC concentration. SOD activity increased significantly in the experimental group of 5.0 mg/L Zn (II). However, with the introduction of OTC, SOD activity decreased, especially to 72.6 U/g in the experimental group of 5.0 mg/L Zn(II) + 5000 μg/L OTC. This implied that in the presence of OTC, it promoted SOD activity of *Coelastrella* sp. at low concentration and inhibited it at high concentration, which was similar to the results of the effect of antibiotics on microalgae SOD activity reported by Aderemi et al. (2018). At the same time, high concentration of Zn(II) can also promote the increase of SOD activity, which was similar to the previous research results of Hamed et al. (2017). Fig. 6a showed that single of Zn(II) had no obvious effect to SOD activity at 0.50 mg/L, but the composition of 0.50 mg/L Zn(II) and OTC could stimulated SOD activity, however the promoting effect was decreased with the concentration of OTC. Fig. 6b showed that single of Zn(II) could also stimulated SOD activity at 5.0 mg/L, but the composition of 5.0 mg/L Zn(II) and OTC inhibited the SOD activity especially in the group of 5.0 mg/L Zn(II) + 5000 μg/L OTC. This may be due to the emergence of Zn-OTC or the complex of Zn (II) and OTC derivatives in the process of combined stress, and these substances can directly inhibit the activity of SOD enzyme in *Coelastrella* sp., and the OTC chelate with Zn(II) could promote SOD activity of microalgae at low concentrations of OTC and Zn(II), but inhibited at high concentrations.

Glutathione (GSH) is a non-enzymatic antioxidant in microalgae, which is composed of glutamate, glycine and cysteine. It involves cell detoxification, free radical scavenging and cellular immunity (Buayam et al., 2019). Therefore, the content of GSH is an important factor to evaluate the antioxidant capacity of cells. As shown in Fig. 6c, Fig. 6d and Table. 2, OTC can promote the GSH content in *Coelastrella* sp. cells, and had the highest value when the concentration was 500 μg/L. The presence of Zn (II) could also promote the GSH content of *Coelastrella* sp., which was similar to the previous research results of Hamed et al. (2017). *Coelastrella* sp. would increase the synthesis of GSH under external Zn (II) stress to resist the toxicity of Zn (II). In the combined stress of 0.50 mg/L Zn (II) and OTC, the content of GSH first increased and then decreased with the increase of OTC concentration. In the combined stress of 5.0 mg/L Zn (II) and OTC, the content of GSH decreased with the increase of OTC concentration. The experimental results showed that the main factor promoting the increase of GSH is Zn (II), because *Coelastrella* sp. cells need to eliminate the toxicity caused by Zn (II) through synthesizing GSH. When OTC and Zn (II) exist together, it would inhibit the synthesis of GSH by *Coelastrella* sp. cells, and the degree of inhibition increased with the increase of OTC concentration.

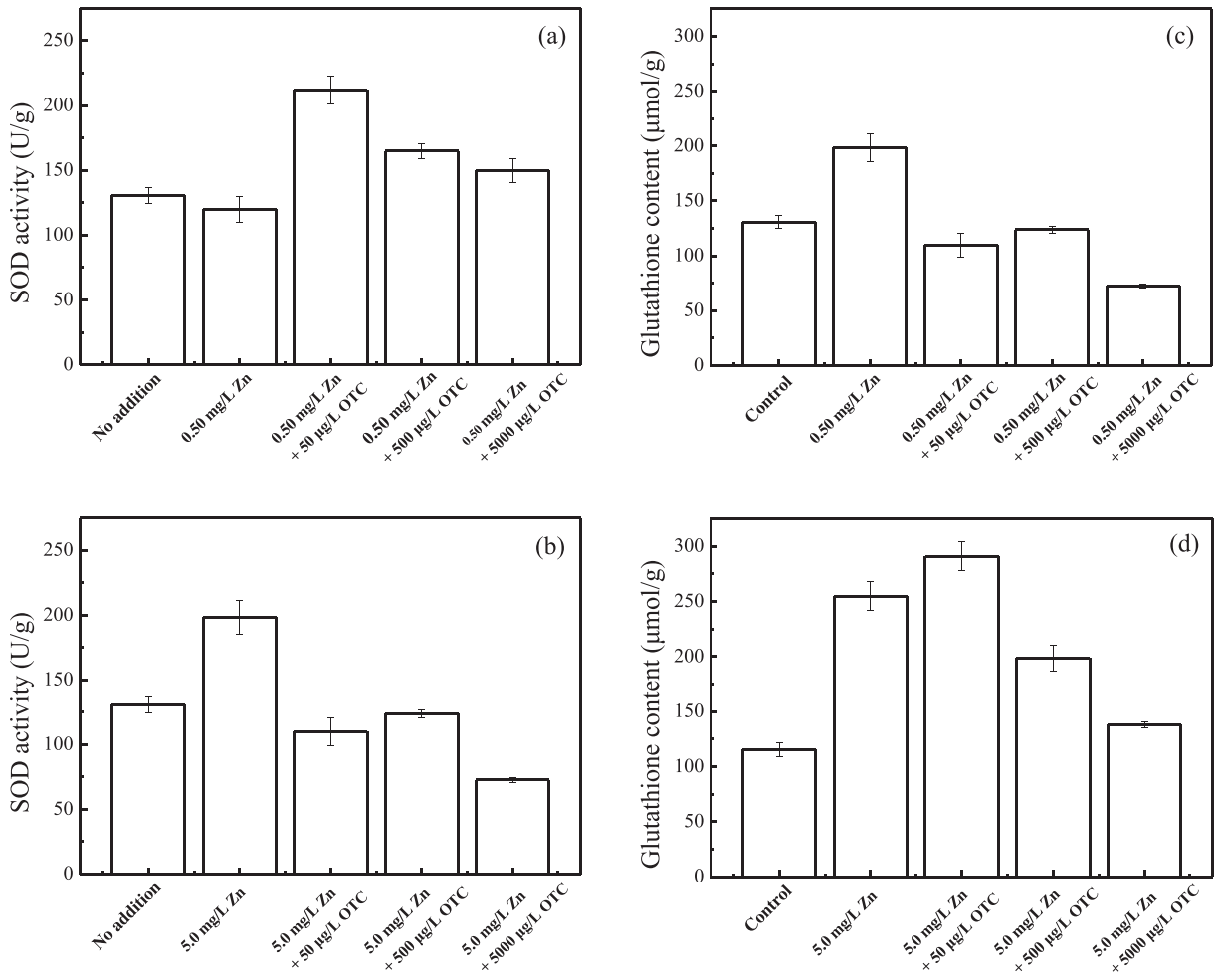

Fig.6. SOD activity and glutathione content of *Coelastrella* sp. at the composition of various concentrations of OTC and constant concentrations of 0.5 and 5.0 mg/L of Zn(II) in SWDE on day 4.

Table 2
SOD and Glutamine synthetase activity, GSH and ATP content of *Coelastrella* sp. at the composition of various concentrations of OTC and constant concentrations of 0.50 and 5.0 mg/L of Zn(II) in SWDE at day 4.

Concentrations		SOD activity (U/g)	GSH content (μmol/g)	GS activity (U/g)	ATP content (nmol/g)
Zn(II) (mg/L)	OTC (μg/L)				
0	0	130.7 ± 6	115.3 ± 6	116.7 ± 6	303.2 ± 16
	50	315.1 ± 22	265.3 ± 18	119.8 ± 8	464.1 ± 34
	500	247.5 ± 3	289.0 ± 22	80.3 ± 6	352.6 ± 18
	5000	163.0 ± 13	207.1 ± 13	57.7 ± 6	583.4 ± 40
0.50		120.0 ± 10	122.3 ± 7	81.5 ± 6	266.2 ± 16
5.0		198.5 ± 13	254.9 ± 3	67.8 ± 4	429.0 ± 33
0.50	50	211.7 ± 11	266.7 ± 21	121.9 ± 8	606.6 ± 46
0.50	500	164.8 ± 6	210.5 ± 23	82.3 ± 4	642.0 ± 32
0.50	5000	149.7 ± 9	142.3 ± 11	58.9 ± 2	687.6 ± 24
5.0	50	109.7 ± 11	291.1 ± 13	91.5 ± 4	811.0 ± 14
5.0	500	123.776 ± 3	198.5 ± 12	81.1 ± 2	711.3 ± 42
5.0	5000	72.5863 ± 2	138.0 ± 3	27.6 ± 1	917.7 ± 23

3.6. Effects of OTC on GS activity and ATP content in microalgae at constant zinc concentrations

Glutamine synthetase (GS) is widely distributed in microalgae and can catalyze the synthesis of glutamine from ammonia and glutamate. It plays an important role in the regulation of nitrogen metabolism in microalgae, especially free ammonia and urea metabolism (Buayam et al., 2019). Therefore, the activity of GS plays an important role in the removal of NH_3-N by microalgae.

As shown in Table 2, the presence of Zn (II) and OTC inhibited the activity of GS in *Coelastrella* sp., and the inhibition efficiency increased with the increase of Zn (II) and OTC concentration. Especially under the combined stress of Zn (II) and OTC, the activity of GS in *Coelastrella* sp. was further inhibited. Combined with the results obtained in Fig. 2a, b and c, it can be found that Zn (II) and OTC reduce the removal efficiency of NH_3-N by microalgae through inhibiting the activity of GS in *Coelastrella* sp., especially under the combined stress of high concentration Zn (II) and OTC.

Adenosine triphosphate (ATP) is the direct source of energy in microalgae. When microalgae form stress response under external stress, a series of proteins and enzymes synthesized will consume ATP, and then affect the removal of TP. As shown in Table 2, when 5.0 m/L Zn(II) and different concentrations of OTC exist alone, they can promote the accumulation of ATP in *Coelastrella* sp. In the combined stress experimental group of 0.5 mg/L Zn(II) and OTC, the ATP content in *Coelastrella* sp. also increased, and increased with the increase of OTC concentration, and further increased in the combined stress experimental group of 5.0 mg/L Zn(II) and OTC, with the highest value in experimental group of 5.0 mg/L Zn(II) + 5000 μg/L OTC. Combined with the changes of lipids and protein contents shown in Fig. 4 and Fig. 5, the reason for the change of ATP in *Coelastrella* sp. might be that *Coelastrella* sp. reduced intracellular toxicity by generating corresponding lipid-containing substances and proteins and chelating with heavy metals and antibiotics. The above process required more energy. Previous studies have

reported that in addition to basic growth consumption, some energy must be used to maintain antioxidant capacity to resist environmental stress (Jia et al., 2019; Letts et al., 2011). This may be used to explain the change trend of TP in Fig. 2. Due to the stress of Zn(II) and OTC, the *Coelastrella* sp. needed ATP, which promoted the assimilation of TP. Therefore, under severe stress, even if the biomass was very low, it still had a good removal effect on TP.

4. Conclusions

The combined stress of OTC and Zn (II) on microalgae inhibited stronger on NH_3-N removal from SWDE than the single stress of OTC. OTC combined with 5.0 mg/L Zn(II) increased the protein content and the accumulation of lipids in *Coelastrella* sp. The presence of 50 μg/L OTC promoted the unsaturation of FAME. With the increase of OTC concentration, SOD activity and GSH content in *Coelastrella* sp. decreased. Zn (II) and OTC in SWDE affected NH_3-N removal via changing biomass production and GS activity, and promoted TP removal due to high accumulation of ATP in *Coelastrella* sp.

CRediT authorship contribution statement

Xiang Li: Conceptualization, Methodology, Software, Visualization, Formal analysis, Investigation, Writing – original draft. **Chunping Yang:** Conceptualization, Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Yan Lin:** Methodology, Software, Data curation, Writing – review & editing, Funding acquisition. **Tianjue Hu:** Investigation, Methodology, Writing – review & editing. **Guangming Zeng:** Conceptualization, Writing - review, Supervision, Resources, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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