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Stress of cupric ion and oxytetracycline in *Chlorella vulgaris* cultured in swine wastewater

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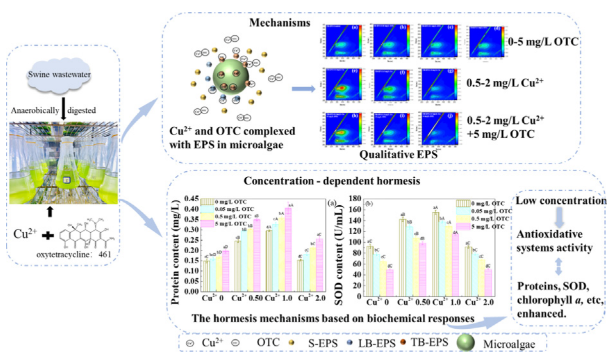
HIGHLIGHTS

- Either OTC or Cu^{2+} induced hormesis in NH_3-N removal via microalgae culturing.
- Lipid content and mass of microalgae maximized when cultured in 0.05 mg/L of OTC.
- Cupric ions induced the oxidative stress response of microalgae in combined stress.
- OTC mitigated the toxicity of Cu^{2+} on microalgae under the combined stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorella culturing has the advantages in treatment of wastewater including swine wastewater from anaerobic digesters due to the product of biolipids and the uptake of carbon dioxide. However, there often exist high concentrations of antibiotics and heavy metals in swine wastewater which could be toxic to chlorella and harmful to the biological systems. This study examined the stress of cupric ion and oxytetracycline (OTC) at various concentrations on the nutrient removal and biomass growth in *Chlorella vulgaris* culturing in swine wastewater from anaerobic digesters, and its biochemical responses were also studied. Results showed that dynamic hormesis of either OTC concentration or cupric ion one on *Chlorella vulgaris* were confirmed separately, and the presence of OTC not only did not limit biomass growth and lipids content of *Chlorella vulgaris* but also could mitigate the toxicity of cupric ion on *Chlorella vulgaris* in combined stress of Cu^{2+} and OTC. Extracellular polymeric substances (EPS) of *Chlorella vulgaris* were used to explain the mechanisms of stress for the first time. The content of proteins and carbohydrates in EPS increased, and the fluorescence spectrum intensity of tightly-bound EPS (TB-EPS) of *Chlorella vulgaris* decreased with increasing concentration of stress because Cu^{2+} and OTC may be chelated with proteins of TB-EPS to form non-fluorescent characteristic chelates. The low concentration of Cu^{2+} (≤ 1.0 mg/L) could enhance the protein content and promote the activity of superoxide dismutase (SOD) while these parameters were decreased drastically under 2.0 mg/L of Cu^{2+}. The activity of adenosine

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triphosphatase (ATPase) and glutathione (GSH) enhanced with the increase of OTC concentration under combined stress. This study helps to comprehend the impact mechanisms of stress on *Chlorella vulgaris* and provides a novel strategy to improve the stability of microalgae systems for wastewater treatment.

1. Introduction

Microalgae can effectively remove and recover nitrogen and phosphorus from various wastewater to synthesize intracellular proteins, lipids, pigments, nucleic acids, and ATPase, particularly in swine wastewater, (Luo et al., 2016; Li et al., 2018; Li et al., 2020b). Microalgae also assimilate CO_2 as inorganic carbon sources and release O_2 to bacteria based on microalgal-bacterial consortium systems, which maximumly removed exceeded 90 % of CO_2 and harvest high concentrations of biomass in high-rate algal ponds (HRAP) (Li et al., 2022d; Serejo et al., 2015; Bahr et al., 2014; Li et al., 2022b).

However, there are abundant heavy metals and antibiotics in swine wastewater, which affected the performances of biological processes for the treatment. Since heavy metals such as Cu^{2+} and Zn^{2+} are also often used as feed additives, >80 % of which are excreted with swine manures and Cu^{2+} at 135–374 mg/kg dry-manure and at 40 mg/L in swine wastewater and Zn^{2+} was detected at 15 mg /L. (Suzuki et al., 2010; Cestonaro do Amaral et al., 2014). Various antibiotics also are commonly used to prevent and treat pig diseases, and promote the growth of food animals as feed additives (Khan et al., 2008), which can be present in livestock wastes. The highest concentration of 2.02 mg/L of OTC and 166.7 mg/kg of tetracyclines (TCs) was detected in swine wastewater and manure, respectively (Ben et al., 2013; Wang et al., 2016).

Heavy metals and antibiotics as stressors induce the hormesis of the biological processes in wastewater treatment expressing a dose-response relationship. At low concentrations, OTC is regarded as a nutrient carbon resource (Wu et al., 2022). Low concentrations of Cu^{2+} and Zn^{2+} can be used as micronutrients to participate material construction of microalgae in the intracellular (Christenson and Sims, 2011). For example, Zn^{2+} can be used as cofactors in the synthesis of proteins and enzyme systems of microalgae cells (Zhou et al., 2018), and Cu^{2+} and Fe are components of photosynthetic electronic operation proteins in microalgae photosynthesis (Li et al., 2020b; Miazek et al., 2015). However, Zn^{2+} will affect microalgae for the absorption of Ca^{2+} at high concentrations, resulting in the activity of ATPase reduction (Liu et al., 2021b). Cu^{2+} replaces Mg^{2+} in chlorophyll, affecting the composition of cytochrome and the transport of photosynthetic electronics, thereby causing the photosynthesis of microalgae to decrease (Xiao et al., 2023). Low concentrations stimulate the synthesis of antioxidant enzymes, antioxidants, protein, and chlorophyll (Xiao et al., 2022), but high concentrations of Cu^{2+}, Zn^{2+}, and antibiotics will cause the accumulation of microalgae reactive oxygen species (ROS) and destroy the balance of the antioxidant system (Nicodemus et al., 2020; Zhou et al., 2018).

Effects of high concentration of Cu^{2+} on the biomass of microalgae and the capacity of nutrients removal and the hormesis induced by Cu^{2+} stress were reported. Liu et al. (2021a) reported that the low concentrations of Cu^{2+} promoted the biomass of *Desmodesmus* sp. CHX1, thus the removal efficiency of Cu^{2+} by microalgae increased. However, when Cu^{2+} concentrations reached 2– 3 mg/L, the growth of microalgae began to be inhibited. Hamed et al. (2017) researched the growth and hormetic effects of two green microalgae in the presence of Cu^{2+}, which induced membrane damage in microalgae, and proline, glutathione levels, the activity of glutathione reductase (GR) and SOD enhanced to mitigate oxidative stress caused by Cu stress. A study by Li et al. (2022b) found that the content of lipids increased when gamma-aminobutyric acid (GABA) combined with copper ions, and hormesis was observed. For example, Cu^{2+} stress significantly up-regulated the content of SOD and peroxidase (POD) for eliminating ROS during the early stage of culturing, subsequently, the antioxidant enzymes activities declined with an increase of culture time. These studies indicated that Cu^{2+} stress could induce the hormesis of microalgae, resulting in performances of nutrients removal of the biological processes affected, and the parameters such as biomass, antioxidant enzymes, and reactive oxygen species in microalgae could be as test endpoints.

OTC belongs to bacteriostatic antibiotics and is one of the tetracyclines. Siedlewicz et al. (2020) reported that the photosynthesis system II of microalgae and cyanobacteria was disrupted, photosynthetic electronics transport was blocked, and photosynthetic pigment synthesis decreased under 8 μg/cm^3 of OTC. A study has reported that the treatment of OTC-containing swine wastewater with *Lemna aequinoctialis* enhanced the synthesis of protein and lutein at low concentrations, but on the contrary at high concentrations, led to increasing intracellular H_2O_2 accumulation, inducing protein carbonylation, and restraining the synthesis of related enzymes in the vitamin *C* synthesis pathway (Hu et al., 2019; Hu et al., 2021). Nevertheless, single contaminants caused the hormesis of microalgae were widely studied, and whether heavy metals and antibiotics can have hormesis on the microalgae treatment process would be studied further.

Microalgae can remove antibiotics via biodegrading and bio-adsorbing (Michelon et al., 2022; Wu et al., 2022). Heavy metals are removed through biosorption and bioaccumulation of microalgae (Hu et al., 2020). However, few studies have reported on the removal mechanism by extracellular polymeric substances (EPS) of microalgae. EPS is a polymer with a variety of functions on the surface of cells, which is also a defense mechanism of microorganisms, and protects microbial cells from dehydration and toxic substances (Sheng et al., 2010; Wu et al., 2022b). The EPS of microorganisms is mainly composed of proteins and carbohydrates, which can adsorb organic and inorganic pollutants due to the binding sites (D'Abzac et al., 2010; Laspidou and Rittmann, 2002). EPS contains a large number of various types of functional groups of aromatic compounds and unsaturated fat chains, which have fluorescent properties, thus, the 3-dimensional excitation-emission matrix (3D-EEM) fluorescence spectrum is often used to study the physicochemical properties of EPS under stress (Sheng et al., 2010). In our study, we questioned whether the mechanisms of Cu^{2+} and OTC on microalgae can be explored and speculated by qualitative and quantitative analysis of EPS.

There are few studies focused on the effects of multiple pollutions on *Chlorella vulgaris* growth and the capacity of wastewater treatment by *Chlorella vulgaris*. In this study, we aimed to reveal the effects of continuous and stable stress of OTC and Cu^{2+} on microalgae processes in swine wastewater, the hormesis in microalgae, and the mechanisms of the stress acting on *Chlorella vulgaris*. The antioxidant stress response of *Chlorella vulgaris* was measured by analyzing the content of SOD, H_2O_2, GHS, and two ATPases. 3D-EEM of EPS and Fourier transform infrared spectrum (FTIR) of microalgae were used to reveal the mechanism of microalgae with OTC and Cu^{2+}. Finally, the intermediates and biodegradation pathways of OTC were proposed.

2. Materials and methods

2.1. Microalgal strain

Chlorella vulgaris comes from Freshwater Algae Culture Collection at the Institute of Hydrobiology, FACHB, Wuhan, China, strain number was FACHB-10, and is cultured in a sterilized BG-11 medium. Table S1 and S2 are the BG-11 media components. The pH of the medium is maintained between 7.0– 7.2 and then sterilized at 121 °C for 15 min. The temperature in the photobioreactor was 25 ± 1 °C, the light conditions were 1000–2000 Lux, and the day/night was 12:12 h.

2.2. Swine wastewater from anaerobic digesters

The experimental wastewater was taken from the anaerobic digester effluent of a swine farm in Hengyang, Hunan, which did not contain OTC and only included a level of μg of Cu^{2+}. The collected swine wastewater was statically settled, centrifuged, and filtered to remove suspended solids. The pretreated was refrigerated at 4 °C. The characteristics of swine wastewater were showed in Table S3.

2.3. Chemicals

$CuSO_4{\cdot}5H_2O$ (Analytically Pure, Shanghai, China) was used as a source of 0.1 g/L of Cu^{2+} in swine wastewater, and oxytetracycline hydrochloride (High Purity Grade) as a source of 0.5 g/L of OTC was derived from Bomei Biotechnology Co., LTD, Hefei, China.

2.4. Experimental design

Three different concentrations of 0.50, 1.0, and 2.0 mg/L of Cu^{2+} were severally combined with three different concentrations of 0.05, 0.5, and 5 mg/L of OTC forming 16 experimental groups, which were set through literature research and pre-experiment. The pretreated swine wastewater was diluted and sterilized and *Chlorella vulgaris* was inoculated in 500 mL of Erlenmeyer flasks with 400 mL of swine wastewater for every experiment group, and the inoculation biomass of *Chlorella vulgaris* was controlled at OD_{680} 0.1. The flasks were placed in a photobioreactor for 12 days of culturing. The culture conditions were consistent with Section 2.1. The flasks were shaken three times every day. And the concentrations of Cu^{2+} and OTC were supplemented every 24 h, to maintain the original concentration level in swine wastewater.

2.5. Chemical analysis

5 mL of solution inoculated algae was centrifuged and filtered for the determination of Cu^{2+} concentration by atomic absorption spectrometry (AAS, Agilent 3510, USA) by Li et al. (2023). The high-performance liquid chromatography (HPLC, Agilent, Palo alto, California, U.S.A.) by Lin et al. (2019) was used to measure OTC, which was detailedly described in supplementary materials.

Chlorella vulgaris biomass was determined by collecting 3 mL of solution using a visible light spectrophotometer (721, Sunny Hengping Scientific Instrument Co., Ltd. Shanghai) at 680 nm, and the dry weight and absorbance of *Chlorella vulgaris* biomass showed a certain linear relationship. Dry weight was calculated as follows equation:

$$DW(g/L) = 0.3225 \times OD_{680}, R^2 = 0.9970$$

Nessler's reagent colorimetric methods by Li et al. (2023) were used to analyze NH_3-N, and TP was analyzed using potassium persulfate digestion methods by Luo et al. (2016).

Lipids extraction from dry biomass of *Chlorella vulgaris* via the Gravimetric method by Zhang et al. (2021a). Microalgae were collected and freeze-dried after 12 days of culturing. 0.1 g of dried biomass was weighed into a 25 mL of spiral tube with 10 mL of a methanol-chloroform mixture (methanol: chloroform = 1:2), and the tubes were ultrasound in an ultrasonic machine for 1 h under the maximum power. Microalgae were extracted overnight for 24 h in a shaker with shaking at 130 rpm, 27 °C. Filtering the extract into a pre-weighed glass tube with a 0.22 μm filter, then 3 mL of ultrapure water was added, rapidly shaken for 1 min, and finally stood for 1 h to remove the upper aqueous phase. The tubes were evaporated at 50 °C in the oven. The change in the weight of the glass tube was the amount of lipids in the dry biomass of *Chlorella vulgaris.* The lipids content was expressed as lipid/dry weight (g/g).

The determination of chlorophyll *a* was determined by Li et al. (2020a). The content of protein and the activity of superoxide dismutase (SOD), glutathione (GSH), hydrogen peroxide (H_2O_2), Ca + +Mg + + -ATPase and Na + +K + + -ATPase were determined by ELISA kit (Shenzhen, China) with 0.1 g microalgae respectively.

2.6. Data analysis

All experimental groups were repeated three times, with results expressed as mean ± standard error (n = 3). IBM SPSS Statistics 26 was used for statistical analyses of data. One-way analysis of variance (ANOVA) and two-way ANOVA were used to determine the significant difference of these parameters under stress, A value of $p < 0.05$ represents the significant difference. All charts were graphed by Origin. Chem Draw 20.0 was used to draw the molecular structures.

3. Results and discussion

3.1. NH_3-N removal by Chlorella vulgaris under stress

NH_3-N is the preferred form of nitrogen utilization by microalgae. The stress of Cu^{2+} and OTC on *Chlorella vulgaris* in the removal of NH_3-N in swine wastewater treatment was followed in Fig. 1. Fig. 1a and e showed the removal efficiency of NH_3-N reached 63.43 %, 65.66 %, 61.11 %, and 57.88 % respectively in the control group and single OTC. It found that in the presence of 0.05 mg/L of OTC, the removal efficiency of NH_3-N was slightly improved in swine wastewater, and within 6– 8 days of *Chlorella vulgaris* culturing, the removal of NH_3-N also was promoted in the single high concentrations of OTC while was reduced subsequently. This result was similar to the growth of *Chlorella vulgaris* and the study by Oliveira et al. (2023). The treatment performance of NH_3-N was optimal by microalgae under the appropriate conditions, which provided the best condition for microalgae growth. It is described as "hormesis". The sub-toxicity of low concentrations stimulates but inhibits at high concentrations in organisms (Erofeeva, 2022). Hormesis is a highly consistent dose-time-response relationship (Agathokleous and Calabrese, 2022). At a low concentration, biomass growth of *Chlorella vulgaris* increased compared to the control group, which could correspondingly promote the removal efficiency of NH_3-N. However, when exceeding adequate concentration, the growth of microalgae began to be inhibited with the culture time increasing. When the single Cu^{2+} was present in Fig. 1b, c, and d, the removal efficiency of NH_3-N was 59.90 %, 54.55 %, and 36.77 %, respectively. The ability of NH_3-N removal by *Chlorella vulgaris* was greatly affected by Cu^{2+} stress. The removal efficiency of NH_3-N was further reduced when Cu^{2+} was combined with OTC. This may be because the content of glutamine synthetase (GS) decreased under combined stress (Fig. S1), which can catalyze glutamic acid and ammonium to synthesize glutamine (Gln) and is also an index for measuring the assimilation level of ammonia nitrogen (Qin et al., 2022). Results showed the hormesis of OTC concentration on *Chlorella vulgaris* in NH_3-N removal processes. The maximum removal efficiency of NH_3-N by *Chlorella vulgaris* was only 65.66 % and its removal ability was slightly inferior to other algae in swine wastewater. This may be the ammonia inhibition due to high initial NH_3-N concentration, which would decrease the growth rate of *Chlorella vulgaris*, and the magnitude of this difference in inhibition is related to our microalgae species (Xia and Murphy, 2016). Higher concentrations of NH_3-N may be toxic to *Chlorella vulgaris.*

3.2. The changes of Cu^{2+} and OTC concentrations in swine wastewater

In order to better study the stress of Cu^{2+} and OTC on microalgae, the trend of the two pollutants in swine wastewater was monitored. Fig. 2 showed that the concentration of Cu^{2+} declined significantly within 2 h, mainly because Cu^{2+} was adsorbed by *Chlorella vulgaris*. The trend of Cu^{2+} concentration was similar to single Cu^{2+} when combined with 0.05 mg/L of OTC (Fig. 2a, b, and c). However, combined with the 0.5 and 5 mg/L of OTC, the Cu^{2+} concentration decreased obviously, which was most pronounced in the group of 2.0 mg/L of Cu^{2+} and 5 mg/L of OTC (Fig. 2c). In all the groups, the concentration of OTC decreased

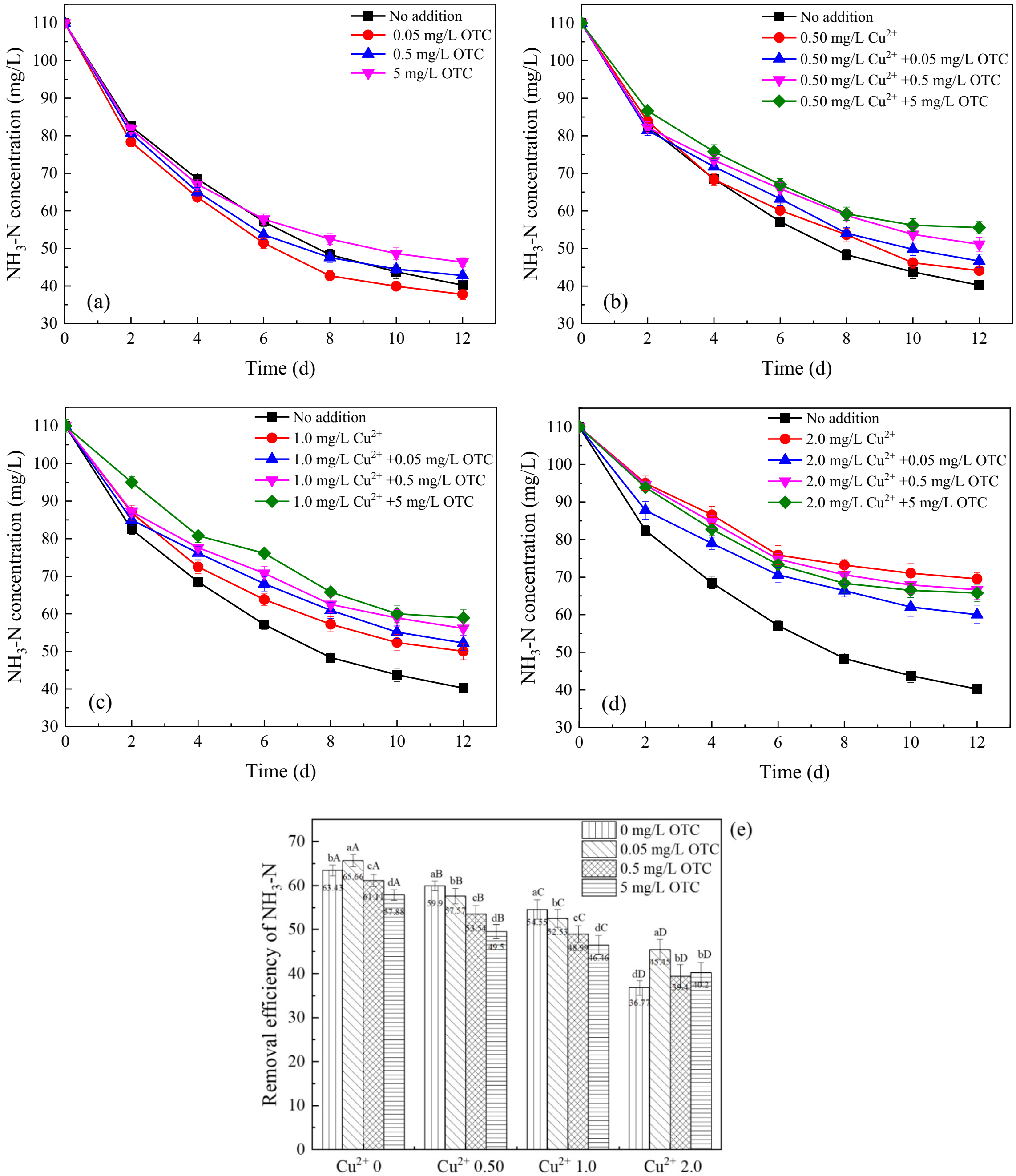

Fig. 1. Stress of different concentrations of Cu^{2+} and OTC on NH_3-N concentration during 12 days of *Chlorella vulgaris* culturing in swine wastewater. (a) 0 mg/L of Cu^{2+}; (b) 0.50 mg/L of Cu^{2+}; (c) 1.0 mg/L of Cu^{2+}; (d) 2.0 mg/L of Cu^{2+}. (e) The removal efficiency of NH_3-N. Error bars are expressed as standard deviation ($n = 3$). Different letters represent that they are different significantly ($p < 0.05$, lowercases represent the same concentration of Cu^{2+}, and uppercases represent the same concentration of OTC).

significantly within 2 h, mainly because of the biodegradation by *Chlorella vulgaris* (Wu et al., 2022). In all the groups of 0.05 mg/L of OTC, the concentration of OTC decreased by >96 % within 24 h (Fig. 2d). Fig. 2e and f showed the higher the concentration of Cu^{2+}, the more OTC concentration decreased, which was similar to the dynamic change of Cu^{2+} mentioned previously. But the decline rate of OTC in swine wastewater slowed down with the increasing culture time, especially in the group of 5 mg/L of OTC the concentration of OTC varied little after 12 h. Results indicated that the change of OTC concentration was greatly affected by Cu^{2+}, which changed significantly at the high concentration of Cu^{2+}. This may be becuse Cu^{2+} have a large potential of complexation affinities with antibiotics including organic ligands, which promotes the adsorption of antibiotics by microalgae through Cu^{2+} bridging effect, similarly, antibiotics can enhance the internalization of Cu^{2+} (Zhao et al., 2013; You et al., 2022).

3.3. Biomass growth of Chlorella vulgaris under stress

Fig. 3a showed during the first 6 days of culture, the presence of OTC promoted the growth of *Chlorella vulgaris* compared to the control group (0.331 g/L). After the sixth day, *Chlorella vulgaris* growth was gradually inhibited in the single OTC of 0.5 and 5 mg/L, and was about 0.312 and 0.294 g/L, respectively. Interestingly, in 0.05 mg/L of OTC, the biomass of *Chlorella vulgaris* was still higher than the control group, and the final

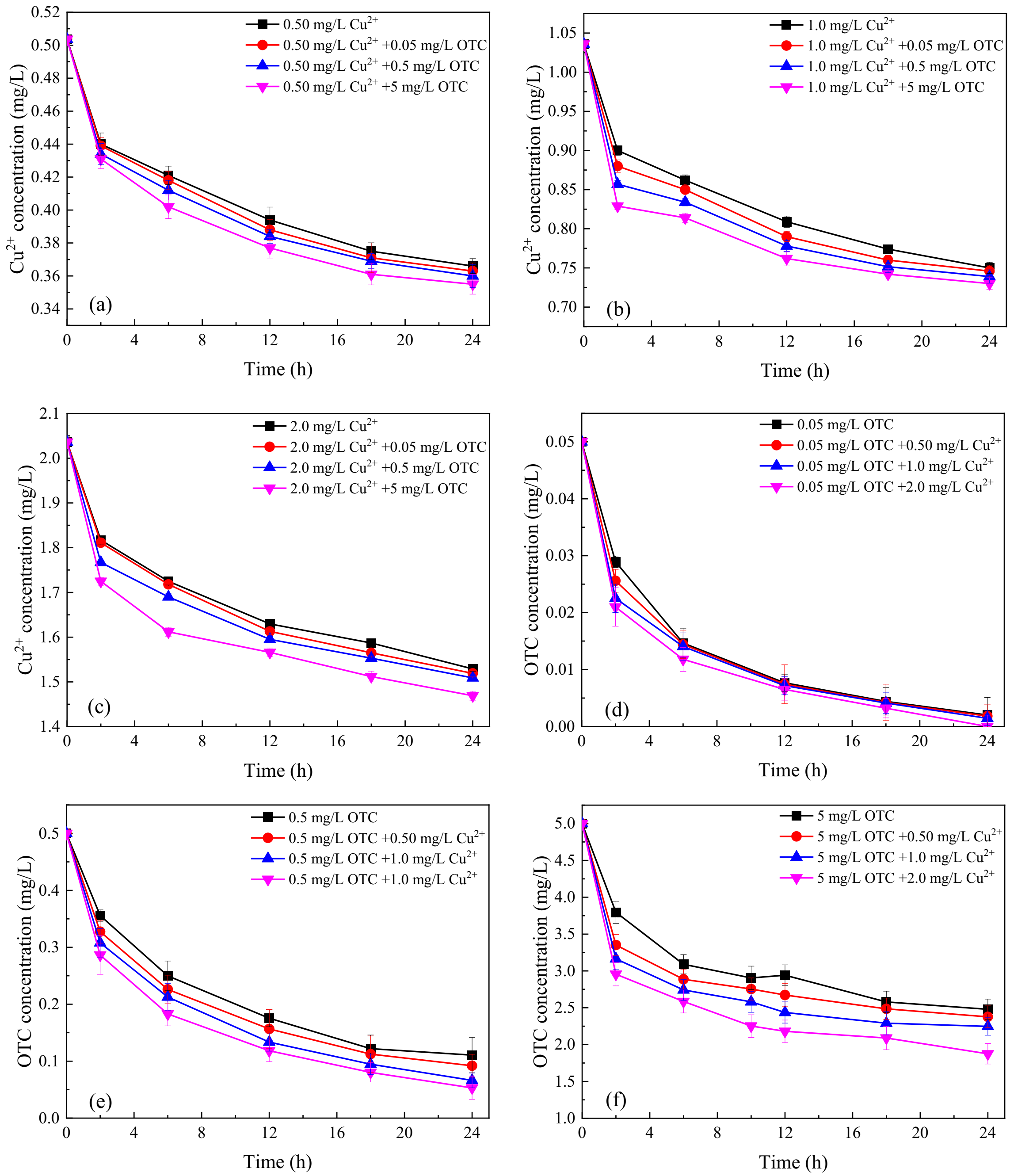

Fig. 2. Changes of Cu^{2+} and OTC concentration at stress of different concentrations of Cu^{2+} and OTC within 24 h of *Chlorella vulgaris* culturing in swine wastewater. (a) 0.50 mg/L of Cu^{2+}; (b) 1.0 mg/L of Cu^{2+}; (c) 2.0 mg/L of Cu^{2+}; (d) 0.05 mg/L of OTC; (e) 0.5 mg/L of OTC; (f) 5 mg/L of OTC. Error bars are expressed as standard deviation (n = 3).

biomass was 0.343 g/L. In Fig. 3b, c, and d, *Chlorella vulgaris* biomass reached 0.288, 0.229, and 0.0780 g/L, respectively, the presence of Cu^{2+} significantly inhibited the growth of *Chlorella vulgaris* biomass. These were similar with previous study. Microalgae were observed significantly hormesis of concentration-dependent growth in the presence of antibiotics and Cu^{2+} could exert greater inhibition on growth (You et al., 2022). OTC may be regarded as a carbon source for *Chlorella vulgaris* at low concentrations which simulated microalgae biomass (Wu et al., 2022). However, in the presence of 0.5 and 5 mg/L of OTC, these revealed the hormesis of concentration-time-dependent relationship. Fig. 3b showed that OTC presented, the inhibition of Cu^{2+} on biomass growth was alleviated, and the biomass yield of *Chlorella vulgaris* maximumly increased by 7.60 % compared with the single Cu^{2+}. The same results were more obvious in Fig. 3c and d. This consistent with the changes of Cu^{2+} and OTC concentrations. In the group of 2.0 mg/L of Cu^{2+} (Fig. 3d), microalgae growth almost stopped from the 4th day, indicating that this concentration of Cu^{2+} expressed greater toxicity on microalgae, and the toxicity was significantly alleviated when 5 mg/L of OTC was added. The inhibition growth induced by Cu^{2+} was alleviated when combined with OTC. This may be because Cu^{2+} firstly complexed with OTC in swine wastewater, when OTC presented, and this detoxification effect was promoted with the concentration of OTC increased, reducing the direct effect of Cu^{2+} on *Chlorella vulgaris* (Wu and He, 2019). Nevertheless, exceeding 2.0 mg/L of Cu^{2+} caused severe and irreversible oxidative stress to microalgae, and combined with OTC to decrease the toxicity on *Chlorella vulgaris* become insignificant.

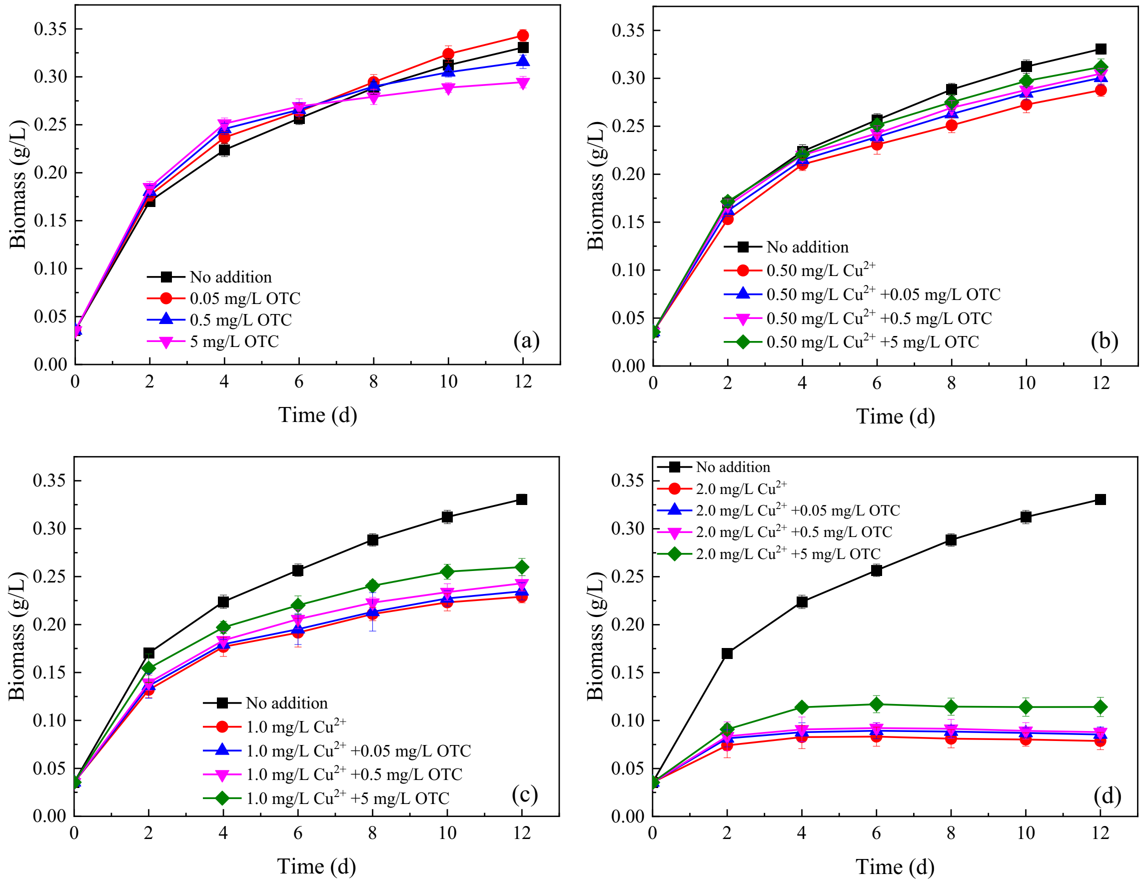

Fig. 3. Stress of different concentrations of Cu^{2+} and OTC on biomass growth during 12 days of *Chlorella vulgaris* culturing in swine wastewater. (a) 0 mg/L of Cu^{2+}; (b) 0.50 mg/L of Cu^{2+}; (c) 1.0 mg/L of Cu^{2+}; (d) 2.0 mg/L of Cu^{2+}. Error bars are expressed as standard deviation (n = 3).

3.4. The content change of protein, lipids, and chlorophyll in Chlorella vulgaris under stress

3.4.1. Protein content

Protein content in microalgae plays an important role in analyzing the hormesis of *Chlorella vulgaris* under stress, including the sum of intracellular proteins and various enzymes. The sub-toxicity at low concentration will stimulate the synthesis of relevant protein to protect the cells from oxidative damage and promote the tolerance of virulence but adverse impact at high concentrations. Fig. 4a showed in low concentrations group of 0.50 and 1.0 mg/L of Cu^{2+}, protein content reached 0.246 and 0.296 g/g, which further increased to 0.351 and 0.405 g/g respectively when 5 mg/L of OTC was added. It showed that long-term exposure of constant low concentrations could induce the hormesis of microalgae, and stimulated the content increase of protein including corresponding plant chelates, metallothionein (Perales-Vela et al., 2006), and some oxidative stress enzymes (Sabatini et al., 2009) for maintaining the *Chlorella vulgaris* growth. Protein content decreased drastically in the group of 2.0 mg/L of Cu^{2+} (Fig. 4a), but similarly, when the concentration of OTC increased, the protein content of microalgae also increased correspondingly. Study proved that Cu^{2+} induced the hormesis of microalgae by stimulation at low concentrations but inhibition at high concentrations, however, the single Cu^{2+} would cause the disorder of the proteomic. Fortunately, CTC combined with Cu^{2+} declined this effect and up-regulated protein, which decreased the toxicity on microalgae (You et al., 2022). OTC and CTC both are belong to the tetracyclines (TCs), thus, the addition of OTC could increase the content of protein and slow the toxicity on *Chlorella vulgaris*. These were similar with the study by (Lu et al., 2015).

Changes in microalgae were induced by exposing to environmental stress such as the content of protein, chlorophyll, and antioxidant enzymes to overcome stress. Unfortunately, excessive concentrations exceeded the tolerance of *Chlorella vulgaris*, and the balance of system of intramolecular was disrupted, resulting ROS accumulating in microalgae, membrane permeability enhancing, and structural and functional proteins being denaturalized and lost (Demidchik et al., 2014; You et al., 2022). Hormesis of microorganism can induce the different levels of survival, resistance, and tolerance to respond higher levels of stressors (Agathokleous et al., 2022).

3.4.2. Lipids content

In Fig. 4b, In the control group, the lipids content of *Chlorella vulgaris* reached 0.423 g/g, which was higher than that of other types of *Chlorella vulgaris* in wastewater treatment (Daneshvar et al., 2018; Kumaran et al., 2023; Shen et al., 2015; Verma et al., 2022). Luo et al. (2016) treated anaerobic digestion of swine wastewater by *Coelastrella* sp., and the lipids content only accounted for 22.40– 25.50 % of dry-weight cells. The high content of lipids made *Chlorella vulgaris* more potential as a raw material of biofuel. The study found that lipids reached a maximum of 0.438 g/g at 0.05 mg/L of OTC, indicating that low concentrations of OTC could not only promote the increase of biomass but also stimulate the accumulation of lipids. In a study by Xie et al. (2019), at concentrations of 1 mg/L of SMX and 5 mg/L of bisphenol A (BPA), the lipids content of *Chlamydomonas* sp. *Tai-0* reached the maximum (25– 30 %), similarly, the lipids content gradually decreased when the pollutant concentration further increased. In the single concentrations of 0.50, 1.0, and 2.0 mg/L of Cu^{2+}, the content of lipids was 0.293, 0.219, and 0.190 g/g, respectively, and the lipids yield was inhibited by 55.08 % maximally. A similar result was reported by Martínez-Macias et al. (2019). This is because Cu^{2+} exerted negative effects on lipids content of microalgae. When Cu^{2+} and 5 mg/L OTC were presented simultaneously, the lipids content reached 0.387, 0.299, and 0.255 g/g, respectively, which increased by 32.53 %, 36.53 %, and

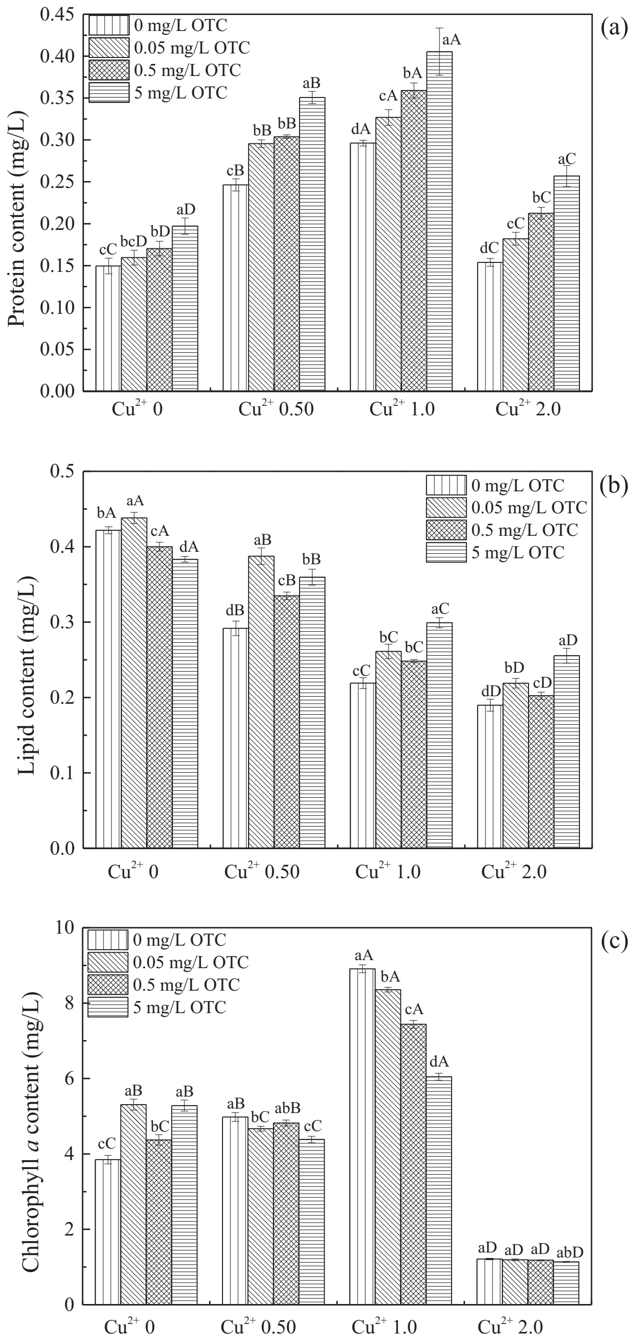

Fig. 4. Stress of different concentrations of Cu^{2+} and OTC on the content of protein, lipids, and chlorophyll *a* during 12 days of *Chlorella vulgaris* culturing in swine wastewater. (a) Protein content; (b) Lipids content; (c) Chlorophyll *a* content. Error bars are expressed as standard deviation (n = 3). Different letters represent that they are different significantly ($p < 0.05$, lowercases represent the same concentration of Cu^{2+}, and uppercases represent the same concentration of OTC).

34.21 % compared with the single Cu^{2+}. The conclusion is similar with Li et al. (2022b). The reason is that combined stress of Cu^{2+} and OTC alleviated the toxicity and up-regulated relevant protein modification and expression, besides, the microalgal glycolysis pathway and the tricarboxylic acid cycle were accelerated, in which the content of ATPase of *Chlorella vulgaris* (Fig. 5d and e), and enzymes related to lipid production (acetyl-Coenzyme carboxylase (ACCase)) were up-regulated, which promote glycogen decomposition and generate related fatty acids (Wang et al., 2021; Xue et al., 2017; Li et al., 2022a; Yang et al., 2023).

3.4.3. Chlorophyll a content

The photosynthetic pigment content of microalgae can be used as a measure of the photosynthetic rate. When microalgae grew under heavy metals stress, Mg^{2+} in chlorophyll molecules could be replaced by heavy metal ions, thereby destroying the chloroplast structure and affecting the photosynthesis process of microalgae (Bechaieb et al., 2016; Xiao et al., 2023). Cu^{2+} is a component of the photosynthetic electronics transporter of microalgae cells, and an appropriate amount of Cu^{2+} was present, which contributed to promoting the synthesis of chlorophyll *a* (Cheng et al., 2019).Compared with other metal ions (such as zinc ions), Cu^{2+} is easier to replace Mg^{2+} in molecules to form copper chlorophyll. What's more, a large number of ROS and H_2O_2 will be accumulated during microalgae photosynthesis under stress, and they cannot be eliminated in chloroplasts in time, the production of chlorophyll will be inhibited (Edreva, 2005).

Fig. 4c showed that at the single OTC, the chlorophyll *a* content increased slightly to 5.31 mg/g. The single 0.50 and 1.0 mg/L of Cu^{2+} promoted the content of chlorophyll *a* in *Chlorella vulgaris*, and the content was about 4.98 and 8.91 mg/g, respectively., on the contrary, the chlorophyll *a* content decreased by 68.57 % in the single 2.0 mg/L of Cu^{2+}. The significant hormesis of microalgae in the aspect of chlorophyll *a* production induced by exposing to Cu^{2+} stress. The low concentrations of Cu^{2+} enhanced the synthesis of chlorophyll *a* and promoted photosynthetic efficiency. When the concentration of Cu^{2+} was excessive, H_2O_2 was accumulated in *Chlorella vulgaris* cells and broke the normal biological function in microalgae (Zhang et al., 2020). When Cu^{2+} was combined with OTC, the chlorophyll *a* content decreased significantly ($p < 0.05$). The combined stress of Cu^{2+} and OTC caused lipid peroxidation of the chloroplast membrane, and the synthesis pathway of chlorophyll *a* was intercepted (Chu et al., 2023).

3.5. Antioxidant stress response under stress

Hormesis is explained protective mechanisms of microalgae, is an overcompensation in oxidative stress response at low concentrations but oxidative damage under excessive stress, and is also the balance of ROS and the synthesis and decomposition of antioxidant enzymes and non-enzymatic antioxidants. Antioxidant enzymes could be as test endpoints of hormesis. (Erofeeva, 2022; Li et al., 2023).

H_2O_2 is a type of ROS, and its content can measure the level of oxidative damage of microalgae under stress (Danouche et al., 2020). The change of H_2O_2 content was consistent with the results of biomass growth of *Chlorella vulgaris.* In Fig. 5a, 0.05– 5 mg/L of OTC did not cause H_2O_2 accumulation, and *Chlorella vulgaris* could regard OTC as a nutrient carbon source for absorption and utilization. The H_2O_2 content was 1.19 μmol/g fresh weight in the control group, and with the concentration increase of the single Cu^{2+}, the content of H_2O_2 increased. The hormetic effect induced by adequate Cu^{2+} stimulated the accumulation of H_2O_2, which was eliminated by antioxidant enzymes. At 2.0 mg/L of Cu^{2+}, the content reached 2.18 μmol/g fresh weight, and the cumulative of H_2O_2 increased by 83.19 % compared with the control group. In the high environmental stress, a large of H_2O_2 generated the balance of the synthesis and decomposition of enzymes being destroyed, resulting in peroxidative damage of microalgae, and the overcompensation became insignificant. It also was proved that Cu^{2+} plays a dominant role in stress of Cu^{2+} and OTC again. Less H_2O_2 was accumulated in combined stress compared with the single Cu^{2+} groups. The reason is that Cu^{2+} complexed with OTC in swine wastewater, besides, combined stress of Cu^{2+} and OTC could decrease the toxicity on *Chlorella vulgaris.* The variation in H_2O_2 content was similar to Danouche et al. (2020) and Chen et al. (2022).

SOD is the first defensive line of the antioxidant defense system in microalgae, catalyze superoxide anions to produce H_2O_2 and O_2, and H_2O_2 is then catalyzed by other enzymes to H_2O and O_2 to prevent microalgae from oxidative damage (Mishra et al., 2021). Fig. 5b showed that the activity of SOD increased with the increase of Cu^{2+} concentrations, subsequently, it subsequently decreased drastically under 2.0 mg/L of OTC.

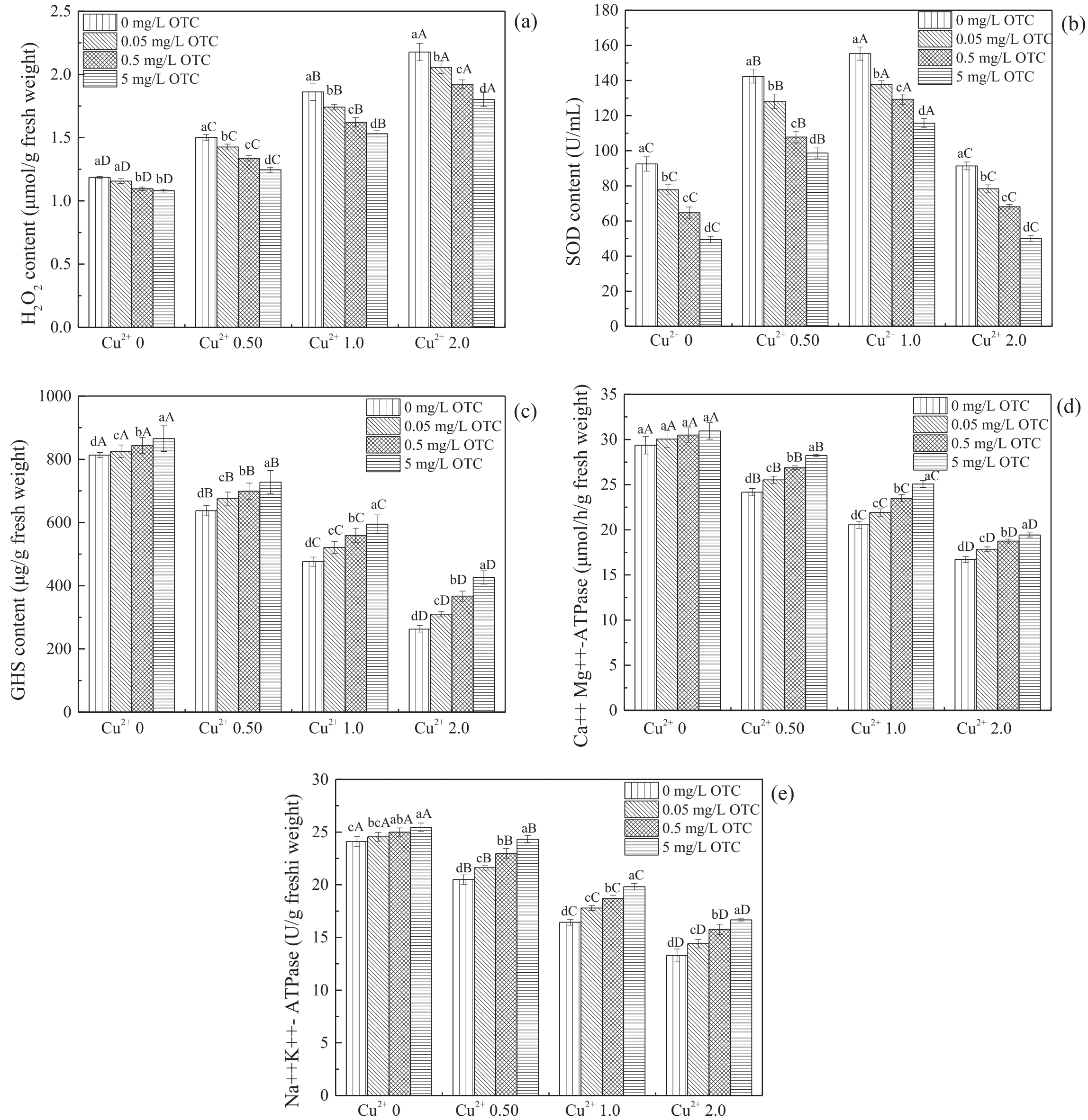

Fig. 5. Stress of different concentrations of Cu^{2+} and OTC on antioxidant stress response of *Chlorella vulgaris* during 7 days culturing in swine wastewater. (a) H_2O_2 content; (b) SOD content; (c) GHS content; (d) Ca + + Mg + + -ATPase activity; (e) Na + + K + + -ATPase activity. Error bars are expressed as standard deviation (n = 3). Different letters represent that they are different significantly ($p < 0.05$, lowercases represent the same concentration of Cu^{2+}, and uppercases represent the same concentration of OTC).

At low concentrations, the antioxidant enzymes were activated for eliminating ROS, while excessive stress induced SOD activities decreasing. This was similar with the study reported by Cheng et al. (2018). Environmental stress would induce the activity of antioxidant defense system, however, excessive stress levels damaged severely the balance of system. Under combined stress of Cu^{2+} and OTC a declining activity of SOD compared with the single Cu^{2+} was observed. This is because Cu^{2+} combined with OTC, a declining toxic of Cu^{2+} impacted on *Chlorella vulgaris*, which decreased the damage induced by Cu^{2+} to the antioxidant system of microalgae. Thus, it could be speculated that the hormesis caused by combined stress would promote the tolerance and resistance of microalgae to environmental stress.

Glutathione (GHS) is a non-enzymatic water-soluble antioxidant in microalgae containing a group of sulfhydryl (-SH) that is complexed with heavy metals (Noctor et al., 1998). GHS can eliminate free radicals of ROS, even directly counteract some ROS, or act as a cofactor and substrate in enzymatic reactions to control ROS levels (Okamoto et al., 2001). For example, at high levels of ROS under environmental stress, with GR enzymes catalyzing, GSH was converted to Glutathione (Oxidized) (GSSG) in the cytoplasm (Upadhyay et al., 2016). Fig. 5c showed that in the presence of the single Cu^{2+}, the content of GHS gradually decreased, and it decreased by 67.70 % in the group of 2.0 mg/L of Cu^{2+}, but in stress of Cu^{2+} and OTC, the consumption of GHS began to decrease. The reason is that the presence of OTC reduced the content of Cu^{2+} via bioaccumulation into the cells.

Ca + + Mg + + -ATPase and Na + + K + + -ATPase catalyze ATP hydrolysis to produce ADP and inorganic phosphorus. The activity of ATPase is determined by the amount of inorganic phosphorus produced (Fig. 5d and e). In the single OTC, ATPase activity increased, because of the degrading of OTC by microalgae. With the single Cu^{2+} concentration rising, the ATPase activity was inhibited, but it gradually enhanced under combined stress of Cu^{2+} and OTC, especially in the group of Cu^{2+} and a high concentration of OTC. The reason is that combined stress upregulated the modification and expression of proteins and various metabolic pathways, in which consumed a number of ATP. Under combined stress, the antioxidant capacity of microalgae was enhanced, and the antioxidant enzymes were activated. Moreover, as pollutants increased, microalgae consume ATP to secrete more EPS, thus the ATPase activity also was enhanced.

3.6. 3D-EEM of EPS and FTIR analysis

3D-EEM and FTIR are often combined to qualitatively analyze the potential mechanisms by which microorganisms remove heavy metals and antibiotics (Zeeshan et al., 2022). The 3D-EEM spectra of three types of EPS in *Chlorella vulgaris* were extracted. It found that the peaks at 260–280/280–310 (Ex/Em), 260–280/340–360 (Ex/Em), 220–230/280–320 (Ex/Em), 220–230/320–350 (Ex/Em), and 240–250/390–430 (Ex/Em) in the fluorescence spectrum corresponded to tryptophan proteins, aromatic proteins, and humus compounds, respectively (Cui et al., 2021). In S-EPS (Fig. S4A), the fluorescence intensity of each peak enhanced as the stress increased, and the position of the peaks gradually redshifted. This may be that *Chlorella vulgaris* exposed to environmental pressure, the content of proteins and carbohydrates in EPS increased (Fig. S2 and S3) to protect cells from damage (Joshi and Juwarkar, 2009; Wang et al., 2015). Moreover, when environmental stress rose, the growth of microalgae was inhibited, and the increased excretion of microalgae cell due to cells shed and lysed could also be manifested as the fluorescence intensity enhanced in S-EPS (Laspidou and Rittmann, 2002). A study by Oliveira et al. (2023) reported that in high concentration of Zn (25–70 mg/L) with increasing culture time, CODs increased in wastewater were attributed to the increasing secretion of EPS (in the form of carbohydrates), and a strong linear correlation between CODs and EPS was proven. This is a strategy to decline the toxicity on microalgae. In LB-EPS (Fig. S4B), with the concentration of single pollution increased, the intensity of the peaks gradually enhanced. When combined stress of Cu^{2+} and the high concentration of OTC, the fluorescence intensity was weaker. In high concentration, a new peak at 230/300–320 (Ex/Em) appeared, and the main peak in LB-EPS also gradually redshifted. The redshift of the peak indicated that the peak corresponded to the material being more active, which was conducive to the growth of microalgae under environmental pressure (Tang et al., 2020). In TB-EPS (Fig. 6), the fluorescence intensity of each peak gradually declined when the concentration of a single pollutant increased, and further weakened in combined stress. The peak at 260–280/340–360 (Ex/Em) corresponded tryptophan protein gradually disappeared. The fluorescence intensity of TB-EPS weakened, because the content of proteins and carbohydrates increased, and the abundant functional groups (amino and carboxyl groups, etc.) of the proteins may be integrated with Cu^{2+} and OTC to produce non-fluorescent chelate (Zhou et al., 2019), particularly the group of Cu^{2+} complexed with 5 mg/L of OTC.

By scanning the FTIR spectrum of *Chlorella vulgaris* that ranged from 400 to 4000 cm^{-1}, the change of functional groups in microalgae was observed (Table 1 and Fig. S5). The multiple spectral bands of these groups have changed in microalgae under stress in experimental group. The peaks at 3305 cm^{-1}, 2997–3012 cm^{-1}, 2927 cm^{-1}, and 1723 cm^{-1} corresponded to -NH of protein, fatty acids containing -CH_2, phenolic groups, and carbonyl groups (C=O); and the peaks at 1649–1658 cm^{-1}, 1539–1546 cm^{-1}, 1448–1462 cm^{-1}, 1045–1190 cm^{-1} were the stretch and vibration of -C=O of protein amide I band, -NH and -C-N groups of protein amide II band, -CH_2, -CH_3, and -C-O bonds in lipids and proteins, and C-O-C and C—O bonds of carbohydrates respectively (Cui et al., 2021; Dobrowolski et al., 2017; Liu et al., 2021a; Wei et al., 2017;

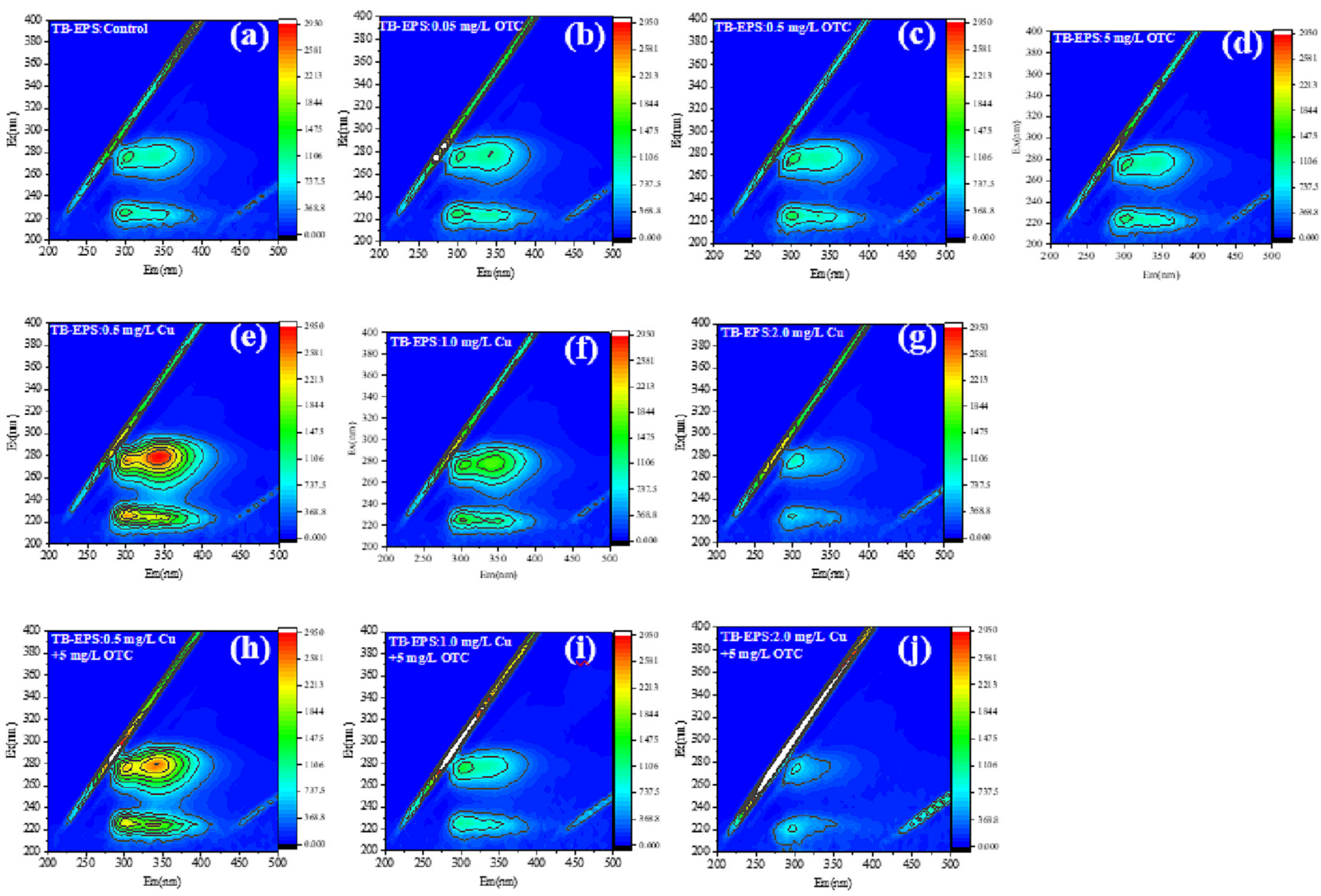

Fig. 6. Stress of different concentrations of Cu^{2+} and OTC on 3D-EEM spectra of TB-EPS of *Chlorella vulgaris* during 12 days culturing in swine wastewater. (a)TB-EPS (control); (b) TB-EPS 0.05 mg/L OTC; (c) TB-EPS 0.5 mg/L OTC; (d) TB-EPS 5 mg/L OTC; (e) TB-EPS 0.5 mg/L Cu^{2+}; (f) TB-EPS 1.0 mg/L Cu^{2+}; (g) TB-EPS 2.0 mg/L Cu^{2+}; (h) TB-EPS 0.5 mg/L Cu^{2+} + 5 mg/L OTC; (i) TB-EPS 1.0 mg/L Cu^{2+} + 5 mg/L OTC; (j) 2.0 mg/L Cu^{2+} + 5 mg/L OTC.

Table 1
Changes of FTIR spectrum of functional groups in *Chlorella vulgaris*.

Peak				Functional groups
Control	Cu^{2+}	OTC	Cu^{2+} + OTC	
3305	3307	3303	3307	-NH of protein
2297	2299–3012	3001	3001–3012	$-CH_2$ of fatty acids
2927	2925	2927	2930	phenolic groups
1723	1720	1721	1720	carbonyl group(C=O)
1651–1656	1649–1656	1649–1656	1649–1658	-C=O of the protein amide I band
1535–1546	1535–1543	1535–1242	1535–1343	-NH of protein amide II and -CN groups
1448–1462	1446–1460	1448–1462	1448–1452	$-CH_2$, $-CH_3$, and -C-O bonds in lipids and proteins
1045–1190	1045–1188	1045–1190	1045–1182	C-O-C and C—O bonds of carbohydrates

Wei et al., 2019). The carboxyl group (-C=O) in the protein made the surface acidic of microalgae and easily expressed a negative charge, which contributed to the adsorption of Cu^{2+} and OTC. These proteins and carbohydrates of EPS were also significantly hydrophobic, further promoting the bioabsorption by *Chlorella vulgaris*. The stretch and oscillation of these peaks indicated that Cu^{2+} and OTC interacted with functional groups of proteins and carbohydrates in the EPS of microalgae. Liu et al. (2021a) reported that after adsorbing Cu^{2+}, the C═O and C═N groups of microalgae at 1644 cm stretched and vibrated, causing the position of the amide I band to change. Moreover, its protein conception changed significantly due to Cu^{2+} mainly complexed with carboxy, hydroxyl, and amino groups of TB-EPS. Zeeshan et al. (2022) found that 30 mg/L of antiviral drugs (AVDs) oseltamivir (OT) stimulated the secretion of EPS in *Chlorella vulgaris. C·S-N* and OT were adsorbed and degraded by interacting with C—H groups in carbohydrates, and -NH and -C=O in proteins of EPS.

3.7. Degradation pathways of OTC

Oxytetracycline was degraded by *Chlorella vulgaris* and the intermediates were measured by LC-MS (Fig. S6). The intermediates of OTC were shown in Table S4 and the possible degradation pathways were proposed. Pathway one in the single OTC, OTC (*m/z* 461) generated m/z 445 by separating the hydroxide group under the action of the intracellular enzyme of *Chlorella vulgaris*, on the one hand, m/z 445 generated m/z 362 by demethylation, dehydroxylation, amino separation, and acylamino eliminating; on the other hand, m/z 445 generated m/z 274 by benzene ring opening, acylamino separating, and hydroxylation, etc., then further dehydroxylation and decarboxylation to generate m/z 262, and finally through benzene ring opening, hydroxyl group and methyl decoupling to generate m/z 102 (Hu et al., 2019; Li et al., 2022c). Under compound stress, one of the pathways was OTC generated m/z 396.9 by separating three hydroxyl groups and one amino group. Another pathway was the hydroxyl group of OTC separated to form m/z 445, after the acylamino and hydroxyl group detached to form m/z 390, which was degraded through two pathways in turn. One was to generate m/z 256 by carbon ring decarboxylation and splitting decomposition, dehydrogenation and dehydration, and finally, benzene ring cracking to generate m/z 114; the second was hydroxyl isolated, methylation, dehydrogenation, $-N(CH_3)_2$ broken away, and benzene ring directly cleavage to generate m/z 209, and finally produce m/z114 (Lian et al., 2021; Liu et al., 2022). The schematic diagram of the degradation pathway of OTC was shown in Fig. 7. Finally, there were five possible complexed forms that Cu^{2+} complexed with OTC in swine wastewater shown in Table S5 (Zhang et al., 2021b; Zhang et al., 2012).

4. Conclusions

Dynamic hormesis of either OTC concentration or Cu^{2+} one on *Chlorella vulgaris* growth were confirmed separately. At appropriate concentration and culture time, OTC promoted the NH_3-N removal, biomass growth, and lipids content of *Chlorella vulgaris.* At the low concentration of Cu^{2+} ($\leq$1.0 mg/L), the content of protein and SOD increased, however, 2.0 mg/L of Cu^{2+} could cause severe inhibition of *Chlorella vulgaris* growth and the accumulation of lipids. Interestingly, the content of lipids, GHS, and activity of ATPase began to increase, and H_2O_2 content decreased under combined stress of Cu^{2+} and OTC, and the presence of OTC helped to alleviate the toxicity caused by Cu^{2+}. Nevertheless, combined stress of Cu^{2+} and OTC expressed insignificant for promoting the removal of NH_3-N. Cu^{2+} and OTC first complexed in swine wastewater, which reduced the toxic effect of Cu^{2+} on *Chlorella vulgaris*. Cu^{2+} and OTC bonded with the

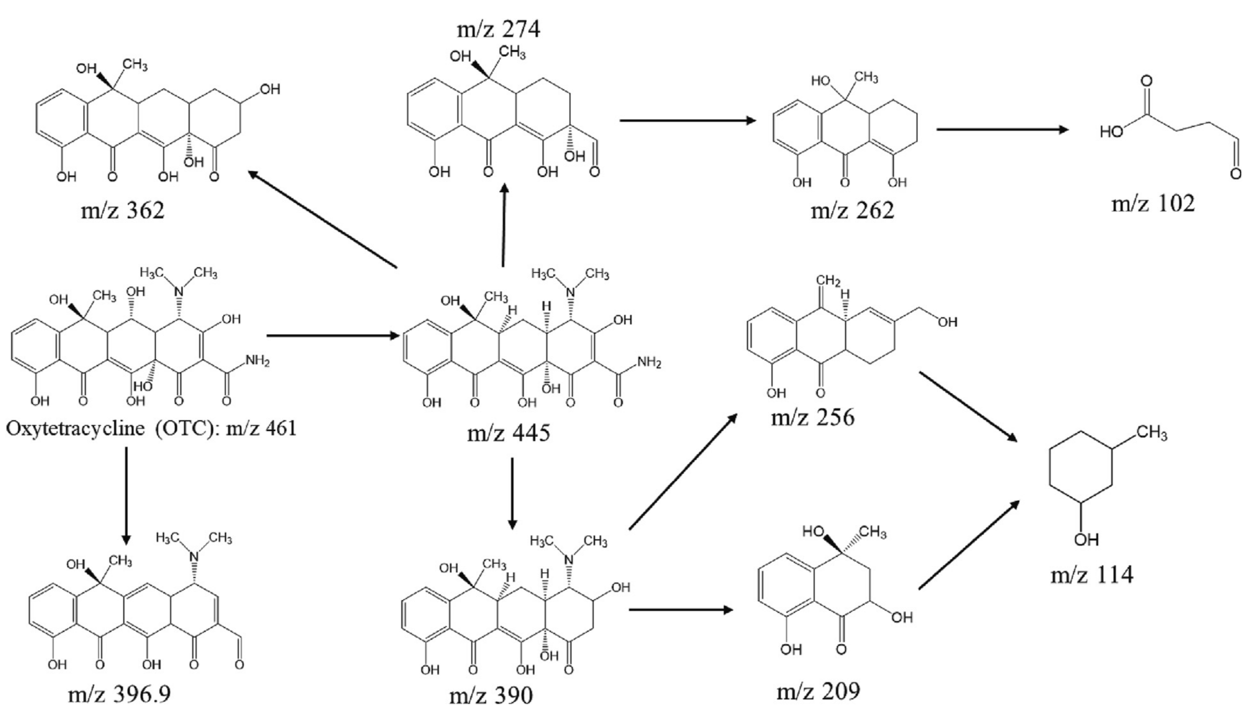

Fig. 7. The schematic diagram of the degradation pathway of OTC.

group of proteins in TB-EPS to produce the chelate of the non-fluorescence characteristics, which promoted mutually adsorbed by *Chlorella vulgaris*.

CRediT authorship contribution statement

Yun Luo: Conceptualization, Methodology, Investigation, Writing-Original draft preparation, Writing-Reviewing and Editing. **Xiang Li:** Conceptualization, Methodology, Investigation, Writing-original draft, Writing-Reviewing and Editing. **Yan Lin**: Conceptualization, Methodology, Data curation, Writing-Original draft preparation, Writing-Reviewing and Editing. **Shaohua Wu**: Visualization, Investigation, Validation, Writing-Original draft preparation. **Jay J. Cheng**: Methodology, Writing-Original draft preparation. **Chunping Yang**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing-Original draft preparation, Writing-Reviewing and Editing.

Data availability

Data will be made available on request.

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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Appendix A. Supplementary data

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