A comparative study of the accumulation and detoxification of copper and zinc in *Chlamydomonas reinhardtii*: The role of extracellular polymeric substances

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Abstract

Extracellular polymeric substances (EPS) form an interface between microalgae and the surrounding water environment. Copper (Cu) and zinc (Zn) are essential micronutrients but may negatively affect microbial growth when their concentrations reach toxic thresholds. However, how EPS affect the accumulation and resistance of Cu and Zn in microalgae remains largely unknown. Here, we investigated EPS production upon Cu/Zn exposure and compared the tolerance strategies to the two metals by *Chlamydomonas reinhardtii* with and without EPS. Microalgal EPS synthesis was induced by Cu/Zn treatments, and the functional groups of polysaccharides and proteins were involved in complexation with metal ions. The extraction of EPS aggravated the toxicity and reduced the removal of metals from solution, but the effect was more pronounced for Cu than for Zn. Copper bound on the cell surface accounted for 54.6 ± 2.0 % of the Cu accumulated by *C. reinhardtii*, whose EPS components strongly correlated with Cu adsorption. In contrast, 74.3 ± 3.0 % of accumulated Zn was absorbed in cells, and glutathione synthesis was significantly induced. Redundancy and linear correlation analyses showed that the polysaccharide, protein and DNA contents in EPS were significantly correlated with Cu accumulation, absorption and adsorption but not with Zn. Data fitted to a Michaelis–Menten model further showed that the EPS-intact cells had higher binding capacity for Cu^{2+} but not for Zn^{2+}. These differential impacts of EPS on Cu/Zn sorption and detoxification contribute to a more comprehensive understanding of the roles of microalgal EPS in the biogeochemical cycle of metals.

Key words: microalgae; accumulation; tolerance; metals; EPS

1. Introduction

Metal pollution in aquatic environments is a major global environmental issue that confronts humanity today and has received much attention (Zhang et al., 2012; Zhou et al., 2020; Ubando et al., 2012). Copper (Cu) and zinc (Zn) are among the most important elements in the metabolic processes of living organisms. Cu is a cofactor in several redox reactions for many metalloenzymes in biological metabolic activities, but excessive Cu can negatively affect the growth of microalgae (such as photosynthetic system damage and cell growth inhibition) and even lead to cell death (Baumann et al., 2009; King et al., 2015; Fawaz et al., 2018). Similarly, Zn is an essential element and plays a key role in the maintenance of normal cell metabolism and development of the microbial immune system; however, high levels of Zn may cause lipid peroxidation and pose a serious threat to organisms (Aravantinou et al., 2017; Chasapis et al., 2012; Zhou et al., 2018). It was reported that the concentration ranges of Cu and Zn in typical swine wastewater (SW) in China were 1.92~5.78 mg/L and 1.30~9.25 mg/L, respectively (Guo et al., 2018; Wang et al., 2021). Since metals are infinitely persistent and can accumulate through the food chain (Ritter et al., 2008; Deng et al. 2016; Cairns et al. 2020), the contamination of Cu and Zn in water has led to environmental and health problems (Rehman et al., 2018; Gong et al., 2011; Xu et al., 2016).

Microalgae, as a type of unicellular or multicellular photosynthetic microorganism, can grow in both aquatic and terrestrial habitats under appropriate conditions (Yin et al., 2019; Junior et al., 2020; Liu et al., 2016). Full understanding of the metal biogeochemical cycle of metals requires the examination of metal interactions with microalgae (Li et al., 2021; Koppel et al., 2017). The mechanisms of metal tolerance in microalgae may be divided into two aspects: 1) prevention of metal ions from entering cells (extracellular binding or exclusion); 2) reduction of intracellular activities of metal ions (formation of metal complexes) (Perales-Vela et al., 2006). Therefore, microalgae have evolved a series of defense mechanisms that alter the fate of metals in a variety of surrounding conditions (Stauber and Davies, 2000; Li et al., 2021). The accumulation and removal of metals by microalgae mainly includes two stages (Kumar et al., 2015): 1) a non-metabolic, rapid and reversible adsorption process that occurs on the cell surface, in which metal ions bind to the functional groups in cell wall and extracellular polymeric substances (EPS) (Ferraz et al., 2004); 2) a metabolic process that takes place in living cells, including metal homeostasis through membrane transport regulation and intracellular complexation (Haferburg and Kothe, 2007; Priyadarshini et al., 2019; Kochoni et al., 2022). The secretion of EPS from microalgae provides a large pool of binding sites that can modify the speciation of metals in the surrounding environment (Xie et al., 2020; Naveed et al., 2020). It is well known that metals can induce EPS synthesis in microalgae (Kiran and Thanasekaran, 2011; Zhang et al., 2016). For example, *Chlamydomonas reinhardtii* (CC-125, FACHB-265) significantly increased the secretion of polysaccharides and proteins in EPS when exposed to silver nanoparticles (Ag-NP), CuO nanoparticles (CuO-NP) and arsenic (As) (Yin et al., 2020; Jiang et al., 2022; Xu et al., 2022). Additionally, it was reported that extracellular polysaccharides in freshwater diatoms increased when Cu concentrations increased from 0.3 μg/L to 10 μg/L (Gonçalves et al., 2018). Some microalgae (cyanobacteria, *Phormidium autumnale*; diatoms, *Nitzschia palea*; and green algae, *Uronema confervicolum*) secreted more extracellular proteins as Zn exposure concentrations increased and contributed to a greater tolerance to Zn (Loustau et al., 2019). Polysaccharides and proteins in EPS contain a large number of functional groups, such as hydroxyl (-OH), carboxyl (-COOH), and sulfhydryl (-SH), which can adsorb metals through ion exchange, complexation and chelation reactions (Luo et al., 2022; Kushwaha et al.,

2012; Joshi and Juwarkar, 2009). Despite the above progress, how EPS affect extracellular and intracellular Cu/Zn accumulation and detoxification in microalgal cells remains unclear and needs more detailed investigations.

Chlamydomonas reinhardtii has a cellular structure and metabolic pathways similar to those found in higher plants. At the same time, *C. reinhardtii* is often used as a biological indicator of environmental pollutants to study cellular mechanisms under metal stress because of its complete genetic sequence and high sensitivity (Zhang et al., 2020; Schroda, 2004; Islam et al., 2017). This alga is also known to be more tolerant to Zn than to Cu (Islam et al., 2017; Xie et al., 2019; Echeveste et al., 2017). After being exposed to Cu/Zn for 96 h, *C. reinhardtii* FACHB-479 and *C. reinhardtii* wild-type strain 137+ (CCAP 11/32C) were more resistant to Zn (EC50 (median effective concentration) values of 54 and 41 mg/L, respectively), while the Cu-EC50 was only 0.22 and 0.39 mg/L, respectively (Li et al., 2013; Webster et al., 2011). To date, the resistance of *C. reinhardtii* to metal(loid)s has been documented mainly for highly toxic elements (e.g., As, Cd, etc.), compared to the less toxic Cu and Zn. Furthermore, previous studies on the tolerance of *C. reinhardtii* to Cu and Zn have mainly focused on the analysis of enzymatic and genomic changes under Cu/Zn stress (Nowicka et al. 2016; Merchant et al., 2007; Chang et al., 2020) and on the determination of intracellular sulfhydryl, metallothionein (MT) and phytochelatin (PC) (Stoiber et al., 2012; Wang et al., 2017; Tsuji et al., 2002), while the role of EPS in the metal detoxification strategy has not been fully elucidated. Therefore, it is necessary to thoroughly study the impact of EPS on Cu/Zn uptake by *C. reinhardtii* and how different components of EPS affect the accumulation and tolerance of the two metal ions in this microalga.

In this study, we compared the differences in the accumulation and effects of Cu and Zn in *C. reinhardtii* with and without EPS. The objectives of this study were: 1) to determine the effects of Cu and Zn on the growth, morphology and intracellular glutathione (GSH) content of algal cells with and without EPS; 2) to reveal the function of EPS on Cu/Zn adsorption and absorption by microalgae; and 3) to compare and analyze the contribution of different EPS components (such as polysaccharides, proteins, and DNA) to Cu/Zn bioaccumulation and tolerance. Findings of this work may elucidate the role of microalgal EPS in metal detoxification and guide the application of phycoremediation of metal pollution.

2. Materials and methods

2.1. Microalgal culture and growth conditions

The freshwater green microalga *C. reinhardtii* (wild type CC-125) was obtained from the Chlamydomonas Resource Centre (University of Minnesota, St. Paul, MN, USA). The alga was grown in Tris-Acetate-Phosphate (TAP) medium (Yang et al., 2012); the TAP medium chemical composition is detailed in **Table S1**. The cultures were subjected to a 12:12 h day/night cycle with a photosynthetic photon flux output of approximately 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a temperature of 25 ± 2 ℃ and a pH of 7.0 ± 0.1 (adjusted with glacial acetic acid). The TAP medium, flasks and vials were autoclaved (30 min at 121 °C), and algal culture inoculation was carried out under aseptic conditions. Before each batch experiment, the microalgal species were aseptically inoculated and cultured for 2~3 cycles and grown to the exponential growth phase to ensure that the microalgal physiology was in good condition.

2.2. Extraction of EPS from *C. reinhardtii*

In this study, we followed the previously published EDTA extraction method to obtain EPS-free *C. reinhardtii* without affecting cell activity (Li et al., 2021; Yu and Fein, 2016). Briefly, axenic

cultures of *C. reinhardtii* cells in exponential growth phase were inoculated into 200 mL of fresh TAP medium with an initial cell optical density at 680 nm (OD_{680}) of approximately 0.06 (corresponding to a cell number of 10^5 cells/mL). After 96 h of precultivation (time required to reach the stationary phase), 180 mL algal cells were centrifuged at 5000 × g for 5 min to remove the supernatant. The algal pellets were resuspended in 20 mL of 0.85 % NaCl solution three times to remove residual culture media and maintain osmotic balance to prevent cell disruption. Then, the suspension was centrifuged again, and an equal volume of 1 mM EDTA solution was added to the algal pellets and shaken for 3 h at 180 rpm. The supernatant collected after high-speed centrifugation (11,000 × g, 15 min) was filtered with a 0.22 μm acetate cellulose membrane to remove microalgal cells and other residues. The filtrate was considered an EPS-containing solution and was stored at -60 °C until analyzed. Finally, the algal pellet was resuspended in fresh sterile TAP medium and considered *C. reinhardtii* without EPS.

2.3. Cu and Zn toxicity to *C. reinhardtii* with and without EPS

The Cu and Zn stock solutions (1000 mg/L) were prepared by dissolving analytical grade $CuSO_4 \cdot 5H_2O$ (0.3929 g) and $ZnSO_4 \cdot 7H_2O$ (0.4396 g) in 100 mL of 18.25 MΩ·cm ultrapure water, filtered through a 0.22 μm acetate membrane and kept in the refrigerator at 4 °C. The influence of EPS on the toxic effects of Cu/Zn was investigated by comparing the growth of algal cells with or without EPS. To do so, a microalgal culture was divided in two, one was used as is while the other was treated to remove EPS. Then 200 mL of *C. reinhardtii* cell suspension with and without EPS were inoculated into 500 mL conical flasks, which contained Cu or Zn at concentrations of 0.5, 1, 3, 5, 7, 10 or 15 mg/L. The culture medium solution without metal ion and with/without EPS were used as controls.

The $OD_{680\ nm}$ of *C. reinhardtii* was measured with a spectrophotometer (MV-06403; SpectraMax) at 0, 24, 48, 72, and 96 h. The relative growth rate (μ) of *C. reinhardtii* in the 96 h inoculation period was calculated using Eq. (1) and Eq. (2).

$$K_e = \frac{\ln OD_{t2} - \ln OD_{t1}}{t2 - t1}, K_C = \frac{\ln OD_{t2} - \ln OD_{t1}}{t2 - t1} \quad (1)$$

$$\mu = \frac{K_e}{K_c} \times 100\% \quad (2)$$

where OD_{t1} and OD_{t2} represent the OD_{680} values of the algal suspensions at 0 and 96 h, respectively. K_c and K_e represent the algal growth rates in the control and treatment groups, respectively, while μ is the growth rate relative to the control.

The results were calculated by fitting S dose-effect curves (Eq. (3)) with Origin software (Version 2022).

$$BR = BR_{\min} + \frac{BR_{\max} - BR_{\min}}{1 + 10^{(\log EC50 - BR) \times Hillslope}} \quad (3)$$

where BR represents biological effects and BR_{min} and BR_{max} correspond to the minimum and maximum biological effects, respectively. The EC50 is the metal concentration (mg/L) value that leads to a 50 % reduction in the cell growth rate of *C. reinhardtii,* and the Hillslope is the slope of the dose–response curve (Karadjova et al., 2017).

2.4. Electron microscopy and attenuated total reflection infrared spectroscopy analyses

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to observe the morphology of algal cells exposed to Cu or Zn with and without EPS. The differences in the surface functional groups were analyzed by attenuated total reflection infrared spectroscopy

(ATR-IR). For this purpose, *C. reinhardtii* were grown to the exponential growth phase and incubated in 200 mL of 5 mg/L Cu- or Zn-containing medium (based on EC50 results), and the microalgae were collected by centrifugation after 4 days (4000 × g, 10 min). Aliquots of the cultures were fixed with 2.5 % glutaraldehyde at 25 °C for 4 h and observed with an electron microscope. Another set of aliquots was freeze-dried and then analyzed by infrared spectroscopy. The measurement method is described in the Supplementary Material (SM) section.

2.5. Characterization of EPS components under Cu and Zn treatments

To assess EPS synthesis by cells under metal exposure, we conducted a batch experiment in which a series of metal concentrations were set up according to toxicity test results. *Chlamydomonas reinhardtii* in the exponential growth phase was inoculated into fresh sterile TAP medium containing 0, 0.1, 0.5, 1, 3 and 5 mg/L Cu or Zn for 4 days, and then EPS was extracted by the EDTA extraction method described above. The EPS were analyzed in terms of chemical composition and total yield to determine the effects of metals on microalgal EPS. The EPS contents in polysaccharides, proteins, and DNA were measured with the phenol sulfuric acid method, the modified Bradford method, and the diphenylamine colorimetric method using glucose, bovine serum albumin (BSA), and calf thymus DNA (Sigma) as standards, respectively (Yuan et al., 2011; Frølund et al., 1996; Burton, 1956). In addition, three-dimensional excitation–emission fluorescence spectroscopy (3D-EEM) and X-ray photoelectron spectroscopy (XPS) analysis of the obtained EPS could further clarify the binding mechanisms of EPS and metal ions. The analytical methods are described in the Supplementary Material (SM) section.

2.6. Removal and bioaccumulation of Cu and Zn by *C. reinhardtii* with and without EPS

To investigate the role of EPS in metal accumulation by *C. reinhardtii*, the following experiments were performed. First, *C. reinhardtii* without EPS was obtained by the EDTA extraction method. Next, the microalgal cells with and without EPS were resuspended in 200 mL of TAP medium (25 °C, pH = 7.0) containing 0, 0.1, 0.5, 1, 3 and 5 mg/L Cu or Zn. After 96 h of incubation, 20 mL of supernatant was collected after centrifugation (5000 × g; 5 min) and filtered through a 0.22 μm acetate membrane. The metal concentrations in the medium were determined by inductively coupled plasma–optical emission spectrometry (ICP–OES, PerkinElmer Optima 8000, USA) to determine the impact of EPS extraction on the removal of Cu and Zn from solution. The relative removal of metals by microalgae is expressed as given below in Eq. (4).

$$\text{Metal removal } \% = \frac{C0-Ce}{C0} \times 100 \quad (4)$$

where *Co* and *Ce* are the 0 and 96 h concentrations of metals in solution (mg/L), respectively.

To quantify the amount of metals bound to the algal cell surface, the procedure described by Hassler et al. (2004) was used. One part of the cell pellet was washed three times with a 5 mM EDTA and 0.85 % NaCl solution (which can effectively remove metal ions bound to the cell surface), while the other part was only washed with 0.85 % NaCl solution to remove excess Cu or Zn from the medium. The freeze-dried algal samples were digested with 3 mL of ultrapure HNO_3, and the solution was heated at 120 °C until colorless (Abboud and Wilkinson, 2013; Worms and Wilkinson, 2007). Finally, the metal contents were determined by ICP–OES. The difference between these two parts was defined as the adsorption of Cu and Zn to the outer surface of *C. reinhardtii*. After Cu or Zn exposures for 96 h, the final pH of the solution was determined, and the concentration of free metal ion species in solution were calculated using MINEQL+ 5.0 (**Table S2, Table S3**). Furthermore, the bioaccumulation data were fitted to a Michaelis–Menten model (Eq. (5)) using the

calculated free metal ions in the media based on media composition and measured pH values at the end of the incubation (**Fig. S1, Table S4**).

$$Metal\ uptake = \frac{V_{max} \cdot K_M \cdot [M^{2+}]}{1 + K_M \cdot [M^{2+}]} \quad (5)$$

where V_{max} is the maximum binding capacity (mg/g DW) of the cells and K_M is the binding affinity for the metal (binding constant) (François et al., 2007).

2.7. Determination of glutathione (GSH) measurements

The intracellular GSH content in microalgal cells exposed to metals with and without EPS was determined using a commercial assay kit (GSH-1-W, Cominbio, China). Each sample was analyzed using the following protocol. In brief, 1 mL of reagent I (extraction buffer) was added to the same volume of algal solution (cells: 5×10^6), and the cells were ultrasonically disrupted in an ice bath (power, 300 W; ultrasound, 3 s; interval, 7 s; and total time, 3 min). The supernatant was collected after centrifugation at 8000 × g for 10 min and filtered using a 0.22 μm filter membrane. Then, 100 μL of ultrapure water, 700 μL of reagent II (assay buffer) and 200 μL of reagent III (chromogen) were added in sequence to 1 mL of the test solution and mixed. After a 2-min waiting period, the absorbance of the solution was measured at 412 nm by spectrophotometry (Molescular Devices, USA) (Wang et al., 2017).

2.8. Data analysis

All experiments were performed in triplicate. Data treatment and analyses were performed using Origin (Version 2022), SigmaPlot (Version 12.5) and SPSS Statistics softwares (Version 25.0). The significant differences ($p < 0.05$) between the control and treatments were determined using one-way analysis of variance (ANOVA, Duncan).

3. Results

3.1. Effects of Cu and Zn on the growth of *C. reinhardtii* with and without EPS

The growth of *C. reinhardtii* with and without EPS showed a significant decline ($p < 0.05$) when the Cu/Zn concentrations were higher or equal to 5 mg/L (**Fig. 1**). By fitting the dose–response curves of exposure to different concentrations of metals on the relative growth rates, the EC50 values (± standard deviation) of *C. reinhardtii* with and without EPS were 10.20 ± 0.21 and 7.28 ± 0.27 mg Cu/L and 8.70 ± 0.12 and 7.04 ± 0.19 mg Zn/L, respectively (**Table 1, Fig. S2**). The growth of microalgae after EPS extraction was thus further inhibited by the two metals, with reductions in EC50 values of 28.6 ± 3.4 and 19.1 ± 2.6 % for Cu and Zn, respectively, indicating that EPS played an important role in the observed tolerance to Cu than to Zn.

Table 1 EC values of Cu and Zn for *C. reinhardtii* with and without EPS after 72 h of exposure.

Metals		Growth inhibition concentration (mg/L)					R^2
		EC10	EC20	EC50	EC80	EC90	
Cu	with EPS	6.51±0.16	7.68±0.11	**10.20±0.21**	13.55±0.56	15.99±0.86	0.9990
	without EPS	3.90±0.25	4.91±0.21	**7.28±0.27**	10.80±0.77	13.59±1.30	0.9968
Zn	with EPS	5.66±0.14	6.64±0.12	**8.70±0.12**	11.40±0.29	13.35±0.46	0.9989
	without EPS	4.98±0.26	5.66±0.21	**7.04±0.19**	8.77±0.40	9.97±0.62	0.9954

Note: the data shown as mean ± standard deviation.

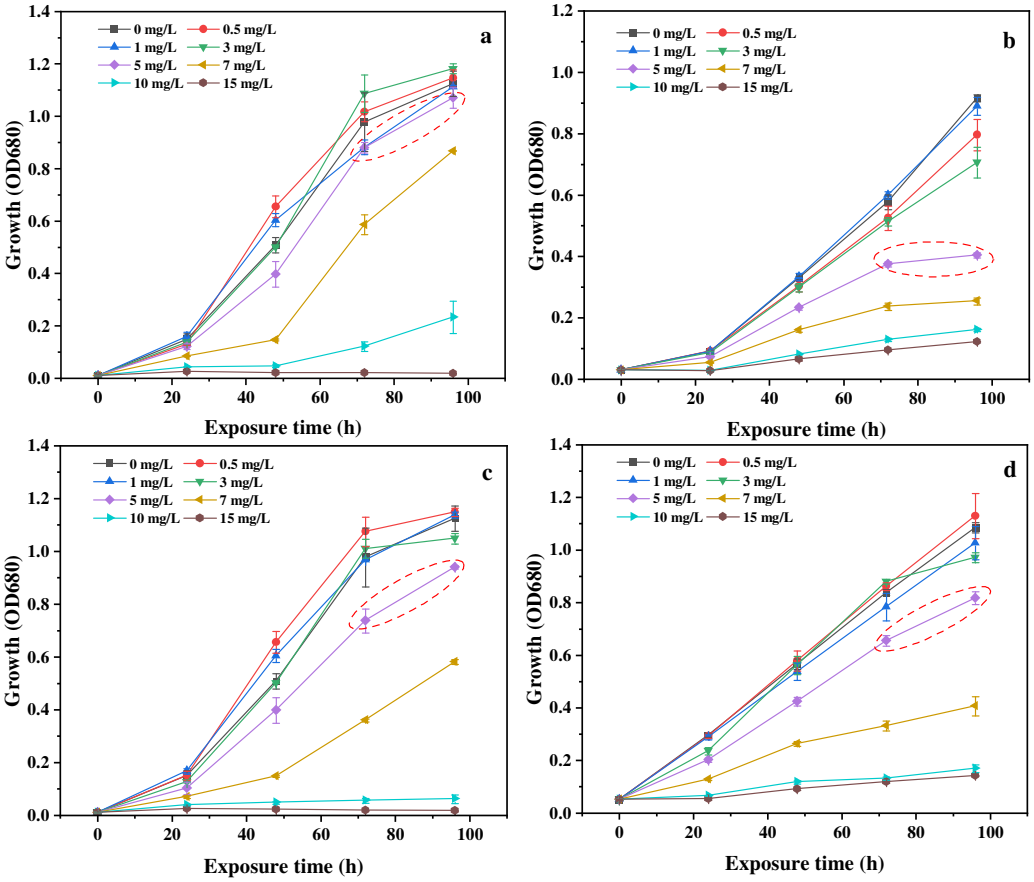

Fig. 1. The optical density of *C. reinhardtii* cultures determined at 680 nm (OD_{680}) with (a, c) and without EPS (b, d) exposed to Cu (a, b) and Zn (c, d) for 96 h. The circled data represent the optical density of microalgal cultures at 72 and 96 h of exposure to 5 mg/L of metals.

3.2. Variation in the compositions of EPS under Cu and Zn stress

The amounts of EPS extracted from *C. reinhardtii* cells exposed to Cu or Zn are shown in **Fig. 2a, b**. The EPS of *C. reinhardtii* extracted by EDTA was dominated by polysaccharides (60.5 ± 5.9 %), which was consistent with the ATR-IR results (**Fig. 2f**). After exogenous additions of Cu or Zn, the contents of total EPS, polysaccharide and protein showed a continuous increasing trend. Compared to the control, the polysaccharide contents increased by 2.4 times for both Cu and Zn, while the protein contents increased by 2.1 times for Cu and 2.5 times for Zn when the metal concentration was 5 mg/L. The protein/polysaccharide ratio for the Zn treatment (0.59 ± 0.06) was higher than that for Cu (0.49 ± 0.01), suggesting that the protein in EPS responded more to Zn exposure, which was also further confirmed by the results presented in **Fig. 2b**. 3D-EEM analysis showed that the fluorescent components in the EPS of *C. reinhardtii* extracted by EDTA had mainly two major peaks, which were located at excitation/emission wavelengths of 280/330 nm (Peak A) and 315/415 nm (Peak B), indicating that the EPS contained tryptophan-like proteins and humic-like substances, respectively (**Fig. 2c-e**) (Chen et al., 2003). The intensity of these peaks significantly increased under Cu/Zn treatment ($p < 0.5$), and the increase in tryptophan-like protein under Cu treatment was significantly higher than that under Zn treatment ($p < 0.5$), while Zn induced more secretion of humic-like substances (**Fig. S3**).

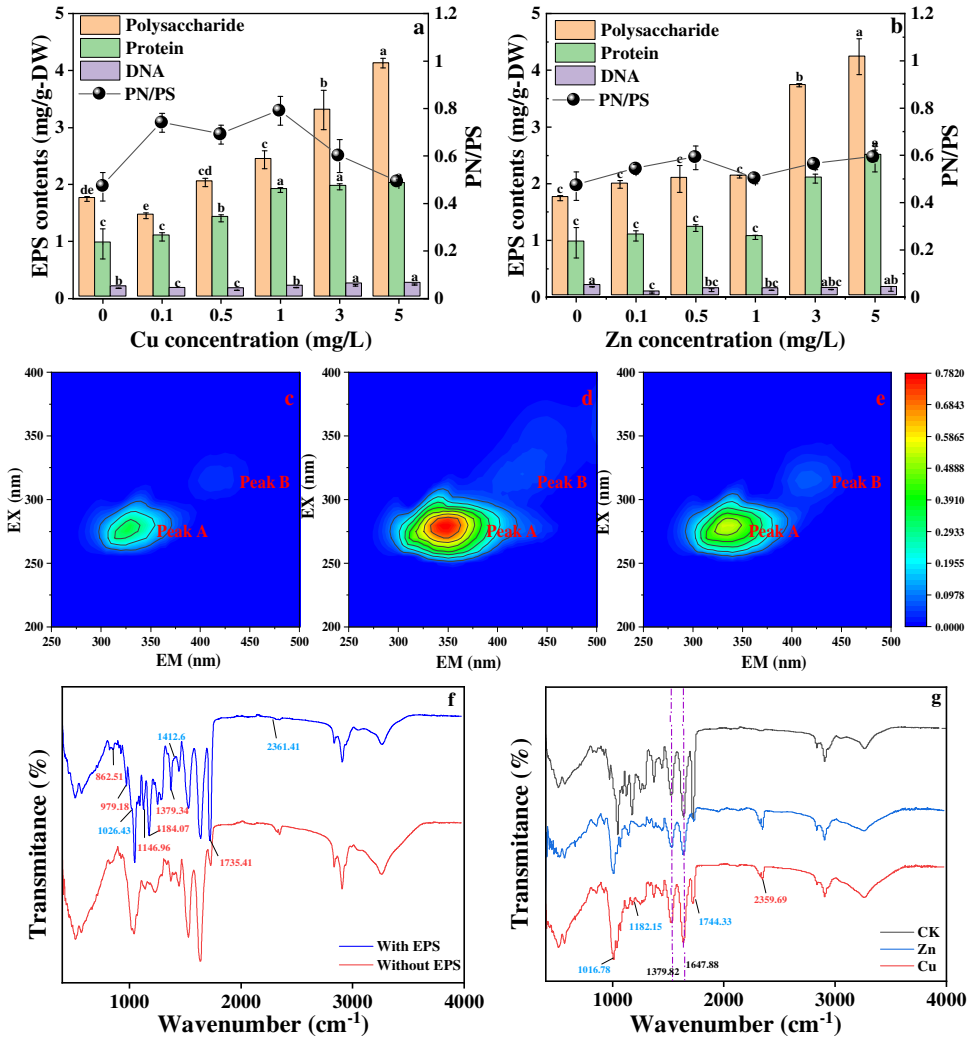

Fig. 2. Distribution of EPS components extracted from C. reinhardtii as a function of Cu (a) and Zn (b) exposure concentrations. PN, protein; and PS, polysaccharides. The bars represent the contents of microalgal EPS components under Cu or Zn treatments; the dots and lines represent the ratios of protein to polysaccharide in EPS; and different letters on the bars indicate significant differences ($p < 0.05$) within a given component over the metal concentration gradient. Each data point indicates the average and standard deviation of three replicates. 3D-EEM fluorescence spectra of EPS from C. reinhardtii without metal exposure (c); with Cu, 5 mg/L (d); and with Zn, 5 mg/L (e). ATR-IR spectra of C. reinhardtii with (control) and without EPS (f) and under different Cu and Zn treatments (g).

Furthermore, XPS analysis was used to characterize the valence-electron changes of metals, carbon, oxygen and nitrogen in EPS (**Fig. 3**). After exposure to 5 mg/L Cu or Zn, distinct metal ion and metal oxide signals were detected in EPS (**Fig. S4**). Through high-resolution scanning of C1s, O1s, and N1s, it was found that the related binding energies characterizing C-O-C (286.19 eV), O-C=O (288.15 eV), C=O (531.15 eV), RC≡N (399.71 eV), and C-NH_2 (401.85 eV) in EPS shifted to a certain extent, indicating that polysaccharides and proteins in EPS were involved in EPS–Cu/Zn binding (**Table S5**).

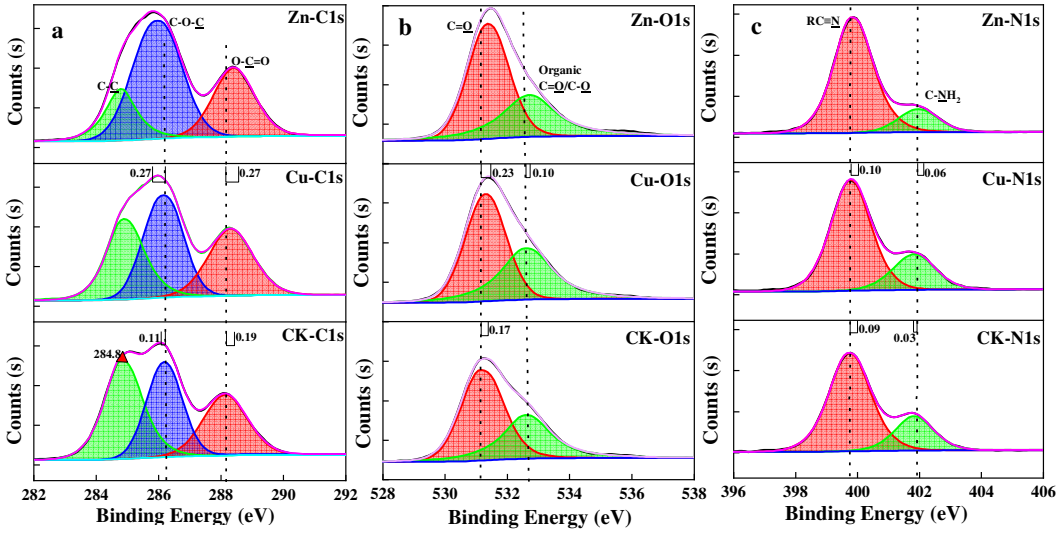

Fig. 3. High-resolution XPS scan of EPS of *C. reinhardtii* after exposure in the absence or presence (5 mg/L) of Cu or Zn. C1s signal (a); O1s signal (b); and N1s signal (c).

3.3. Effects of Cu, Zn and EPS on the cell morphology and surface properties

SEM images revealed that the cell surface of microalgae without EPS was smoother (no obvious covering) than in the presence of EPS (**Fig. 4a, b**). After exposure to 5 mg/L Cu, the cell size of the microalgae increased significantly, and a large amount of EPS was secreted at the cell surface (**Fig. 4c**). However, Zn treatment only had a minor effect on the secretions produced by the cells (**Fig. 4d**). TEM images further showed the changes in the subcellular structure of microalgae after Cu and Zn treatments. Compared to control cells, the intracellular chloroplast layered structure was deformed, the lamellar thylakoid was loose, and the interface between the cell membrane and cell wall was not obvious (**Fig. 4g**), indicating that the microalgal cysts were seriously damaged by Cu. At 5 mg/L Zn, the cell volume did not increase significantly, but the intracellular starch granules increased significantly, and the protoplasts contracted severely (**Fig. 4h**). However, large vesicles with clear structures appeared inside the cells, in which Zn-like aggregates were observed, and EPS-like flocs were found to be secreted outside the cells.

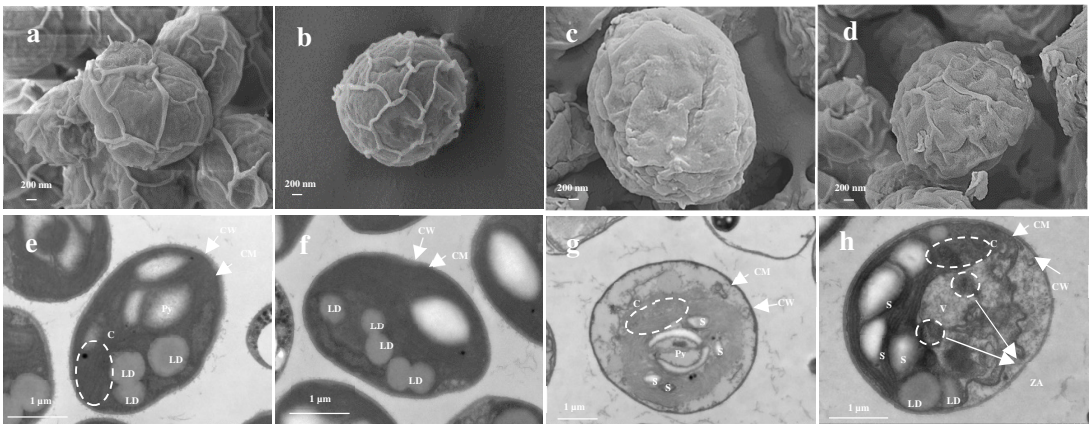

Fig. 4. SEM and TEM images of *C. reinhardtii* of the control (a, e), without-EPS (b, f), cells exposed to 5 mg/L Cu (c, g) and 5 mg/L Zn (d, h). CW: cell wall, CM: cell membrane, Py: pyrenoid, S: starch grains, LD: liquid droplets, V: vacuole, ZA: Zn aggregates, and C: chloroplast.

The ATR-IR spectra showed a decrease in the peak intensity of functional groups on the surface of microalgal cells after EPS extraction (**Fig. 2f, g**), e.g., at 979.18, 1146.96, 1215.90, 1261.70, and 1735.14 cm^{-1} for polysaccharides (C-O, C-O-C and C=O, carboxyl groups (lipids), etc.). The intensity of the functional groups used to characterize proteins only increased at 1379.34 cm^{-1} (amide III) but did not change significantly at 1542.77 (amide II) and 1636.78 cm^{-1} (amide I), indicating that the EDTA-extracted EPS was mainly composed of extracellular polysaccharides (**Fig. 2f**). After exposure to 5 mg/L Cu or Zn, it was observed that the functional groups of both polysaccharides and proteins on the surface of microalgal cells were involved in the binding of metal ions (**Fig. 2g**). After binding to Cu, the peak was shifted to the right at 862.51 cm^{-1}, indicating that C=O (unsaturated bonds) and C-O-P (possibly DNA) were involved in the binding of Cu. At the same time, a significant decrease in peak intensity was observed at 1076.08, 1182.15 and 1647.88 cm^{-1}, suggesting that extracellular polysaccharides were involved in EPS–Cu binding, while the functional groups characterizing proteins only showed a slight decrease at 1379.82 cm^{-1}, implying that polysaccharides in EPS were the main component of EPS–Cu. After exposure to Zn, there was a remarkable decrease in peak intensity mainly at 1379.82, 1437.47 and 1636.78 cm^{-1}, which suggested that the main Zn-binding component of EPS were extracellular proteins.

3.4. Removal of Cu and Zn by the algal cells with and without EPS

To determine whether the metal removal ability of *C. reinhardtii* was affected by EPS, the removal and adsorption capacity of microalgae before and after EPS extraction were compared for each metal (**Fig. S5**). At the highest concentration tested, the concentration reduction and adsorption capacity of Zn by *C. reinhardtii* were 2.51 ± 0.11 mg/L and 3.73 ± 0.09 mg/g, respectively, which were significantly higher than those of Cu (0.71 ± 0.01 mg/L and 2.50 ± 0.13 mg/g, respectively). However, when EPS were removed, the adsorption capacities of microalgae for 5 mg/L Cu and 5 mg/L Zn decreased by 60.0 ± 0.1 % and 4.3 ± 2.5 %, respectively, which suggested that EPS played a more important role in the removal of Cu than Zn (**Fig. S5a, c**). In addition, comparing the contribution of different components of EPS to Cu binding, it was found that the Cu binding per unit of polysaccharides and proteins increased when the concentration was in a range of 0.1 to 3 mg/L (**Table S6**), which may be caused by the secretion of additional EPS from microalgae after extraction (Naveed et al., 2020). However, when the Cu concentration was increased to 5 mg/L, a decrease in Cu binding per unit was observed. In contrast, Zn treatment presented completely different results. The Zn binding per unit of the two EPS components generally decreased with increasing metal concentration in the growth media (**Table S6**).

3.5. Bioaccumulation of Cu and Zn with and without EPS

In Cu-exposed cells without EPS, the maximal accumulation was reduced by 68.5 ± 0.1 % compared to cells with intact EPS. When the Cu treatment was lower than 5 mg/L, the Cu absorption by the microalgae with EPS was higher than the adsorption, but as the concentration increased, the opposite trend was found (**Fig. 5a**). After the removal of EPS, microalgae mainly adsorbed Cu at 0.1 mg/L, while the overall trend did not change (**Fig. 5b**). In contrast to Cu, microalgae exhibited a completely different accumulation pattern for Zn, in which absorption dominated when the Zn concentration was higher than 3 mg/L (**Fig. 5c, d**). More importantly, there was no significant difference in Zn accumulation, adsorption and absorption by *C. reinhardtii* with and without EPS, indicating that EPS did not affect the accumulation process of Zn by microalgae.

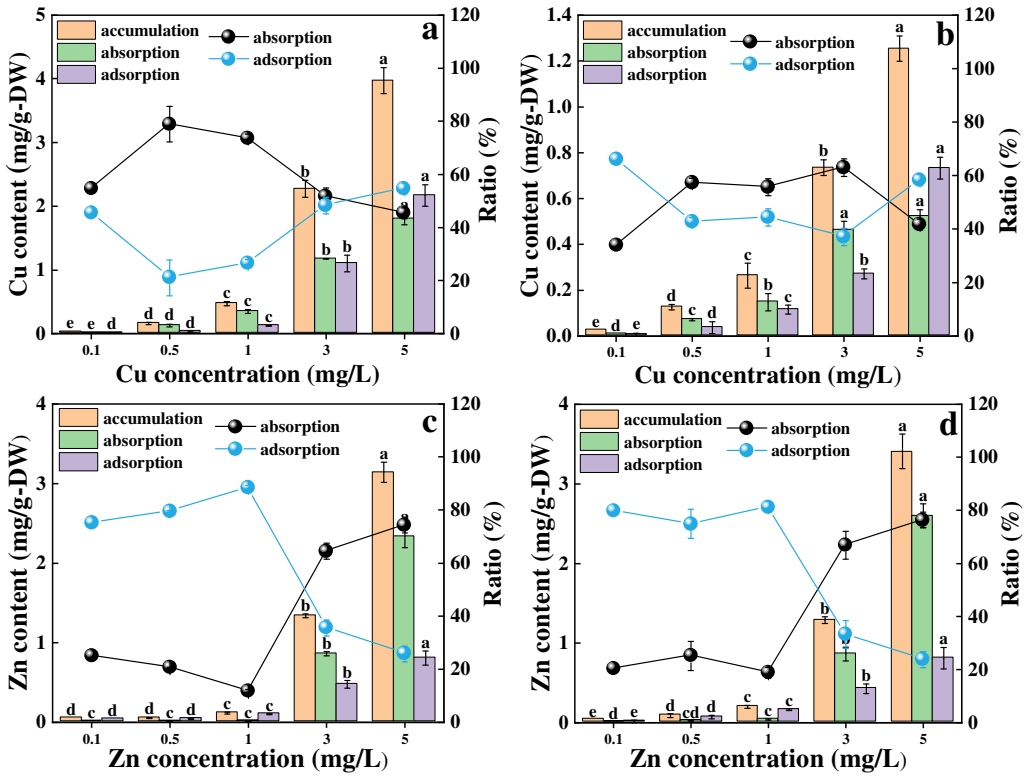

Fig. 5. Metal bioaccumulation (adsorption or absorption) by *C. reinhardtii* with EPS (a, c) and without EPS (b, d) under different Cu and Zn treatments. The bars represent the level of accumulation, absorption and adsorption of Cu and Zn in microalgae; the dots and lines represent the proportion (ratio) of absorption and adsorption to the accumulation; and different letters on the bars indicate significant differences ($p < 0.05$) over the concentration gradient for each series. Each value indicates the average and standard deviation of three replicates.

The Cu profiles showed a biphasic process (**Fig. 6a, b**), and there was a typical saturation of adsorption and absorption as free ion concentrations increased. For absorption, in the presence of EPS, microalgal cells had a greater binding capacity (approximately 4 times) than the results obtained in the absence of EPS. The binding affinity of Cu^{2+} to the absorption sites of cells in the absence of EPS was quite high, with a value of $10^{9.28}$ (standard error range: $10^{9.18}$ ~ $10^{9.36}$) (**Fig. 6a, Table S7**). Similarly, microalgal cells with intact EPS had a higher binding capacity (approximately 2.5 times) in the adsorption process than those without EPS. Additionally, the binding affinity of Cu^{2+} to the adsorption sites of the cells ($10^{7.69}$) was lower than that for absorption sites ($10^{8.09}$) in the presence of EPS, while the best fitted K_M value was not significant in the absence of EPS (**Fig. 6b, Table S7**). In contrast, the Zn profiles indicated no notable differences in absorption and adsorption patterns in the presence or absence of EPS (**Fig. 6c, d**). For adsorption, a slight curvature was observed, but the data distribution did not allow us to extract significant binding constants. On the other hand, significant values for the binding capacities were obtained, and these were not significantly different whether EPS was present or absent, indicating that the presence of EPS did not affect the Zn-binding capacity of the adsorption sites.

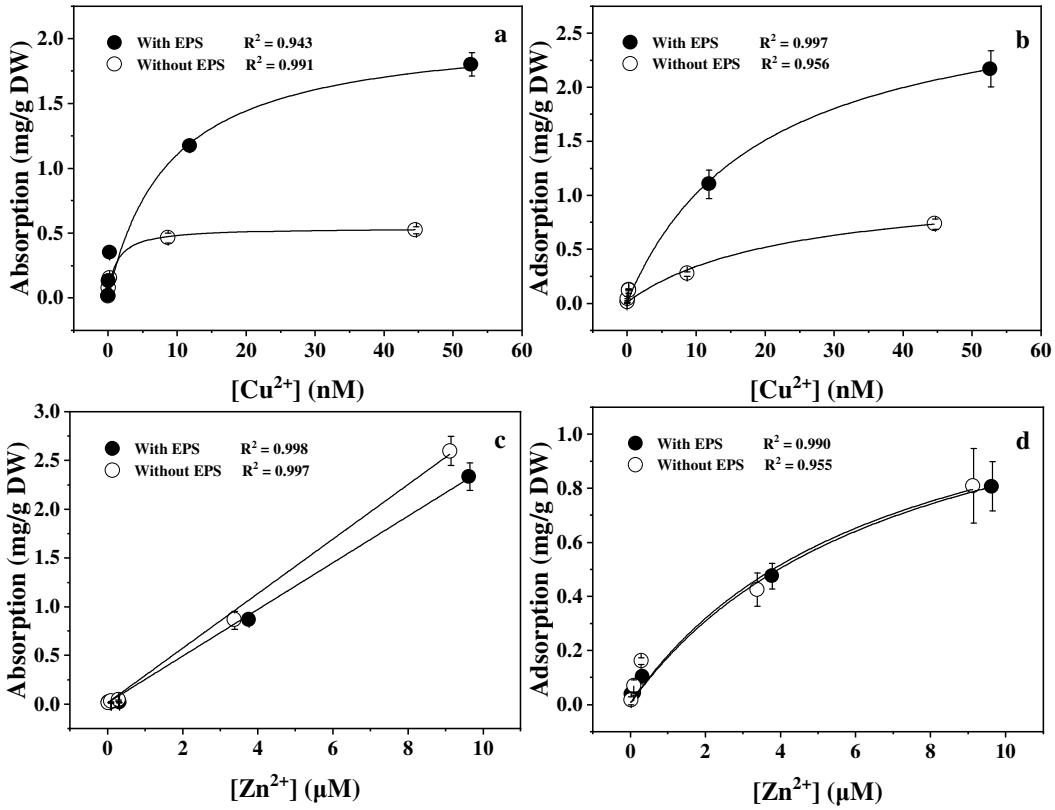

Fig. 6. Absorption (a, c) and adsorption (b, d) as a function of calculated free Cu^{2+} (a, b) and Zn^{2+} (c, d) by *C. reinhardtii* with and without EPS with a fitted Michaelis–Menten model. Representative constants are given in Table S7 in the Supplementary Material.

Through a linear correlation analysis of the accumulation, absorption and adsorption of Cu or Zn with different components of EPS, it could be seen that microalgal polysaccharide, protein and DNA had a significant positive correlation with the sorption process of Cu, while Zn was mostly negatively correlated (**Table S8**). Meanwhile, redundancy analysis further confirmed the results (**Fig. S6**). In addition, the contribution of different components to the bioaccumulation of Cu and Zn by microalgae was further compared (**Table S9**). The effects of polysaccharides and proteins in EPS on Cu sorption increased with Cu concentration, especially under stress (at 5 mg/L). These effects were more significant for Cu adsorption than absorption, which was consistent with the results in **Fig. 4**. However, related results were not obtained for Zn, indicating that EPS did not play a role in the bioaccumulation of Zn by microalgae (**Table S9**).

3.6. Metal induced GSH production of algae with and without EPS

Through the analysis of the GSH content over the tested metal concentration range, the differences in the intracellular response to Cu and Zn by *C. reinhardtii* were clarified (**Fig. 7**). As the Cu concentration increased, the microalgal GSH content before and after EPS extraction was 4.91 ± 0.34~5.09 ± 0.39 mmol/g-DW and 4.91 ± 0.34~6.7 ± 1.8 mmol/g-DW, respectively, with no significant differences, indicating that intracellular GSH complexation was not the major detoxification mechanism of Cu in microalgae. In the Zn-exposed cells, the content of GSH did not change significantly at concentrations below 3 mg/L. However, when the concentration was 3 mg/L and 5 mg/L, the GSH content before and after EPS extraction significantly increased by 3.0 and 11

times and 3.5 and 13 times, respectively, which was consistent with the bioaccumulation process shown in **Fig. 5c, d**. In addition, the GSH content significantly increased after EPS removal and Zn exposure, which could further indicate that EPS removal contributed to increasing the overall metal exposure, allowing more free Zn^{2+} ions to enter cells (**Fig. 6c**).

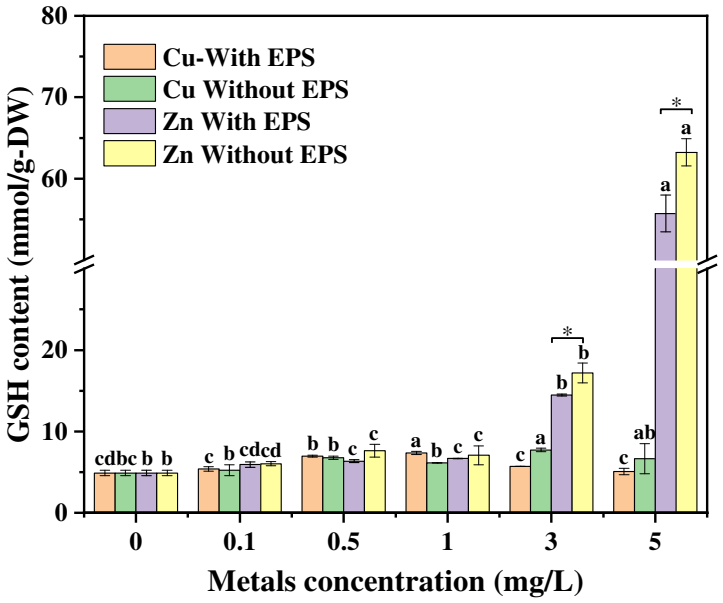

Fig. 7. The glutathione (GSH) contents of *C. reinhardtii* with and without EPS after exposure to Cu or Zn. Different letters on the bars indicate significant differences ($p < 0.05$) among the GSH contents for each individual treatment; and * indicates significant differences ($p < 0.05$) among the GSH contents with and without EPS treatment. Data are means ± SDs (n = 3).

4. Discussion

In microalgal cells, there are a variety of strategies for the resistance against metal toxicity, such as extracellular complexation, intracellular redox reaction, chelation and efflux, gene regulation, and synthesis of antioxidant or antioxidative enzymes (Gómez-Jacinto et al., 2015; Wang et al., 2017). As a typical metabolite, EPS are known to greatly influence the tolerance and bioaccumulation of metals in microalgae (Hou et al., 2013; Naveed et al., 2020; Yang et al., 2022). However, how EPS play a role in the toxicity and accumulation of Cu and Zn in *C. reinhardtii* and how they affect the distribution of metal ions in microalgal cells still need to be elucidated. Therefore, we comparatively evaluated the role of EPS in the toxicity of Cu and Zn to *C. reinhardtii*, analyzed the effects of EPS and different components on metal removal/sorption and revealed the roles of EPS in the cellular response of *C. reinhardtii* to the two metals.

4.1. Effects of Cu and Zn on the growth and cell surface properties of *C. reinhardtii* with and without EPS

In our study, the growth of *C. reinhardtii* was significantly inhibited by the addition of Cu or Zn, and the relative growth rate decreased more considerably with increasing Zn total concentrations than Cu (**Table 1**). Considering that metal complexation is much greater for Cu than for Zn, the expected range of free Zn^{2+} is two to three orders of magnitude greater than that for Cu^{2+} (**Table S4**). When considering metal speciation, the cells show a greater sensitivity to Cu^{2+} than Zn^{2+}. This

is consistent with the literature that shows that the Zn tolerance of different species of *C. reinhardtii* is higher than that of Cu (**Table S10**). For example, Li et al. (2013) and Webster et al. (2011) determined EC50 values for *C. reinhardtii* FACHB-479 and *C. reinhardtii* wild-type strain 137+ (CCAP 11/32C) after 96 h of exposure to Cu and Zn, respectively. The EC50 values were found to be 53.8 and 41.3 mg/L for Zn, respectively, whereas the EC50 values for Cu were much lower than those for Zn at 0.22 and 0.39 mg/L, respectively, indicating that *C. reinhardtii* was more tolerant to Zn. In addition, by summarizing the toxicological data of Cu and Zn on green algae over the past decade (**Table S10**), it was found that for *C. reinhardtii,* most of the EC50 values for Cu were between 0.083 and ~0.39 mg/L (Islam et al. 2017; Wang and Dei, 2006; Webster et al. 2011), which were much lower than our findings (10.1 mg/L). The discrepancy may be caused by the difference in metal speciation, strains and culture conditions (e.g., growth medium composition, light intensity, temperature, etc.).

When the algal EPS was removed using EDTA extraction, the tolerance of *C. reinhardtii* to Cu and Zn changed (**Table 1**). The results agree with those reported by Naveed et al. (2020), who found that the removal of EPS reduced the growth of *Synechocystis* by 49.4 % and 43.7 % with increasing concentrations of As(III) and As(V), respectively. However, by comparing the EC50 values of microalgae with and without EPS to Cu and Zn, it was found that the removal of EPS resulted in a greater sensitivity of *C. reinhardtii* to Cu exposure, indicating that EPS had a higher impact in mitigating the effects of Cu than Zn.

SEM and TEM were applied to visually compare the effects of these two exposure regimes on the morphology and subcellular structure of microalgal cells (**Fig. 4**). The 5 mg/L Cu treatment increased the cell volume, deformed the chloroplast structure and loosened the layered thylakoid (Shanab et al., 2012; Zamani-Ahmadmahmoodi et al., 2020). Similar studies found that, upon exposure to 1 mg/L Cu^{2+} and 100 mg/L CuO NPs, the chloroplast structure of *Chlorella* sp. and *Scenedesmus* sp. was significantly deformed (Che et al., 2018). Similarly, when the green alga *Coccomyxa actinabiotis* was exposed to silver ions, the same phenomenon occurred (Leonardo et al. 2015). The absorption of Cu^{2+} destroyed the chloroplast structure, leading to chlorophyll degradation in microalgal cells. El-Kassas and Okbah (2017) found that Cu inhibited photosynthesis, reduced chlorophyll content, and altered cell morphology and enzyme activity at high concentrations. In addition, Jamers et al. (2013) found that exposure to high concentrations of Cu resulted in more reactive oxygen species (ROS) in cells. ROS production in microalgae is dependent on cell size, with larger cells producing more ROS and showing a direct relationship between cell size and the amount of $O_2^{\bullet-}$ produced (DalCorso, 2012; Ugya et al., 2020; Hansel and Diaz, 2021). Echeveste et al. (2017) found that when *Chlorolobion braunii* was exposed to 3.18 mg/L Cu, the volume of microalgae increased from 116 nm^3 (control) to 283 nm^3. In addition, under metal stress, the number and volume of starch grains in cells increased. This is mainly because microalgae can synthesize starch to store the energy required for physiological activities (Brányiková et al., 2011). For example, intracellular starch granules can be used for energy consumption when synthesizing protein macromers (enzymes and proteins) with greater redox activity and high complexing capacity (Dao et al. 2017; Solovchenko, 2012). Also, under the action of hexokinase, uridine-5-diphosphate (UDP)-glucose pyrophosphorylase, glycosyltransferase and polymerase, starch accumulated during photosynthesis was secreted to the outer layer of cells as a soluble polysaccharide in response to a metal stress (Kumar et al., 2015). Meanwhile, the content of intracellular lipid droplets decreased in metal-exposed cells. It has been found that the lipid synthesis of *C. reinhardtii* is greatly affected

by high concentrations of Cd and Hg, which is caused by lipid peroxidation (Nowicka et al., 2016). Lipids constitute heterogeneous dynamic groups of biomolecules that play a crucial role in the process of microalgal cells withstanding metal stress. They may not only be used as energy sources in chloroplast membranes but can also be antioxidants to maintain redox stability (Moellering and Benning, 2011, Nowicka et al., 2016). In our Zn treatments, no significant changes in cell volume and no visible damage to intracellular organelles were noticed, but Zn-like aggregates were found in intracellular vesicles (**Fig. 4h**). It has been reported that metal-induced chloroplast damage can be monitored by the overexpression of chloroplast heat shock protein HSP70B, which can prevent protein damage under harmful environmental conditions (Chankova et al., 2014). Heide et al. (2009) believed that HSP70B was a key element in the formation of the VESICLE-INDUCING PROTEIN PLASTID 1 complex, which participated in thylakoid biogenesis to maintain the membrane integrity of *Chlamydomonas* under metal stress (Liu et al., 2007). Compared to other metals, Zn could be less toxic (Küpper and Andresen 2016), and its toxicity is mainly due to the substitution of other divalent metal ions, particularly the replacement of Mg^{2+} in chlorophyll, which leads to structural instability when chlorophyll forms protein complexes (Nowicka and Kruk 2016). Mikulic and Beardall (2014) found that the EC50 of *C. reinhardtii* expressed as free Zn^{2+} ion concentration was only 0.038 mg/L. The toxicity of Zn mainly affected pigment production in *C. reinhardtii* cells but did not impair photosystem II activity in microalgal cells and therefore did not affect microalgal organelles (especially chloroplasts). In addition, the cell morphology and subcellular structure of *C. reinhardtii* with and without EPS showed a significant reduction in cell surface coverage and no damage to the cell structure (**Fig. 4**), indicating that EPS extraction by EDTA did not affect the growth of microalgae. It is well known that the microbial cells naturally secrete EPS even if there are no environmental stress (Flemming and Wingender, 2010; Siddharth et al., 2021). After the EPS removal, conditions for rapid regeneration of EPS include: 1) microalgae still maintain good cell activity; 2) growth conditions of microalgae do not change (light, temperature and basic nutrient elements, etc.); 3) microalgae are exposed to non-lethal environmental stresses (metals, organic pollutants and nanoparticles, etc.) (Naveed et al., 2020). In our work, the EDTA method used to extract EPS from microalgae did not cause damage to cells, and the subsequent experiments were carried out using the same culture medium. Therefore, *C. reinhardtii* with EPS extracted had certain re-synthesis of EPS, and with the increase of metal ion concentration, EPS also showed an increasing trend (**Fig. S7**). However, compared with microalgae with EPS (**Fig. 2a, b**), the total amount of re-synthesis is much lower. Similarly, Naveed et al. (2020) also observed EPS re-synthesis in *Synechocystis* PCC 6803 after the removal of EPS and arsenic treatments for 8 days, but the total amount of EPS re-generation was very limited. So far, due to the complexity of EPS, research on the molecular mechanism of EPS synthesis and secretion by green algae under metal treatment is very limited, but these processes have been partially studied in bacteria and cyanobacteria (Zhang et al., 2018). The synthesis mechanisms of extracellular polysaccharide mainly include the following four steps: (1) activation of monosaccharide and conversion of monosaccharide into glyconucleotides in the cytoplasm; (2) assembly of glycosyltransferases on the plasma membrane of repeating units by adding sugar sequence to the lipid carrier; (3) polymerization of repeating units on the plasma surface of the plasma membrane; (4) polymer output to the cell surface (Pereira et al., 2009). Related genes involved in the secretion of extracellular polysaccharides include genes encoding nucleotides and sugar synthetases, glycosyltransferases and polysaccharides processing (Pereira et al., 2009). Glycosyltransferases

play a key role in the transfer of extracellular polysaccharides (Morgan et al., 2013; Schäper et al., 2017). Morgan et al. (2013) found that the complex structures of intimal protein (BcsA) and periplasmic protein (BcsB) play a very important role in the process of exocytosis of polysaccharides. It is also reported that cyclic dimeric GMP (c-di-GMP) can regulate the biosynthesis of microbial EPS (Schäper et al., 2017). However, the specific regulatory mechanism of protein synthesis in EPS under metal treatment remains unclear.

Differences in the functional groups present on the surface of microalgae with and without EPS were shown by using ATR-IR, in which the intensity of the functional groups for polysaccharides (C-O, C-O-C and C=O, carboxyl groups (lipids), etc.) was significantly weaker, indicating that the method was effective for EPS extraction. However, the functional groups associated with proteins showed no significant changes except for an increase in peak intensity at 1379.34 cm^{-1} (amide III) and at 1542.77 cm^{-1} (amide II) and 1636.78 cm^{-1} (amide I), indicating that the protein extraction was not adequate, which was consistent with the EPS compositional analysis (**Fig. 2f, g**). Moreover, Naveed et al. (2020) showed that the removal of EPS did not cause significant differences in the cell surface functional groups, but their peak positions were shifted to a certain extent, as shown by an analysis of infrared spectra after the extraction of EPS from *Synechocystis* PCC 6803 using the heating method (50 °C, 20 min).

4.2. Effects of EPS on the Cu and Zn removal and bioaccumulation by *C. reinhardtii*

In general, there is a consensus that EPS synthesis is one of the main responses to environmental stresses by microalgae (Zhang et al. 2013; Nekvapil et al. 2021; Wu et al. 2010). In this study, it was found that extracellular polysaccharides secreted by *C. reinhardtii* increased by 1.36 and 1.43 times, and proteins increased by 1.10 and 1.60 times, after exogenously addition of Cu or Zn, respectively (**Fig. 2a, b**). It has been reported that after 14 days of Cu stress, the extracellular polysaccharide and protein of the cyanobacterial strain *Lyngbya putealis* increased from 75.4 ± 2.5 and 5.03 ± 0.74 μg/mL to 183.5 ± 3.3 and 18.4 ± 1.3 μg/mL, respectively (Kiran and Thanasekaran, 2011). A similar study also found that after exposure to 12.71 mg/L Cu for 2 weeks, the secretion of extracellular polysaccharides and proteins in *Chlorella* increased by 49 % and 56 %, respectively (Zhang et al., 2013). Jian et al. (2011) found that under the stress of 6 mg/L Cu, *Bacillus vallismortis* cells the highest EPS content was found in the logarithmic phase of growth, and the EPS yield increased from 48.7 to 60.7 mg/g, while for 12 mg/L Zn, the the highest EPS content was found in the stable phase of growth, and the EPS yield peaked at 101 mg/g. As typical macromolecules, EPS can be transferred out of cells by changing the fluidity of cell membrane (exocytosis) upon various stresses (Gao et al., 2020; Pereira et al., 2009; Tian et al., 2022). For example, nanoparticles containing metal ions may significantly induce ROS production and Ca^{2+} signaling in microalgal cells (Liu et al., 2019; Tsai et al., 2018). Under oxidative stress, microalgal cells secrete metabolites such as proline, tryptophan and secondary metabolites such as aminobutyric acid and boric acid, which promote the glycolytic-TCA cycle and provide energy source for EPS secretion. In particular, more threonine is consumed to regulate lipid metabolism, thus enhancing the fluidity of cell membrane and facilitating the secretion of EPS (Salehi et al., 2018; Majdi et al., 2017). In addition, the change of Ca^{2+} concentration is related to the inositol phospholipid pathway (Tsai et al., 2018; Chen et al., 2017). The inositol phospholipid pathway may be used to control a variety of cellular processes, including secretion, cytoskeletal regulation, intracellular vesicle transport, and cell membrane synthesis (Ohta and Suzuki, 2007). Therefore, heavy metal exposure may cause cell membrane damage and further affect inositol phospholipid

metabolism, and then affect the secretion of EPS through Ca^{2+} signal transduction. However, at higher metal concentrations, the secretion of EPS decreased significantly, which may be due to toxic effects of metals, leading to the destruction of cell structure, thus hindering the synthesis and transportation of intracellular sugars and proteins, leading to the decrease of EPS secretion (Grąz et al., 2011). To date, studies on the effects of free Zn ions on microalgal EPS production are limited. Most previous studies have been related to Zn nanoparticles. For example, *Microcystis aeruginosa* increased the yield of LB-EPS (loosely bound EPS) by 46.3 % and TB-EPS (tightly bound EPS) by 2.67 % in the presence of ZnO nanoparticles, and the increase was mainly in polysaccharides compared to proteins and humic-like substances (Hou et al., 2017; Xu and Jiang, 2015). Combined with infrared spectroscopy and XPS, it was further revealed that the functional groups characterizing proteins (C=O, amide I and C-N, N-H, amide II) on the cell surface of microalgae showed a decrease in peak intensity and shift in position at the 5 mg/L Zn exposure level, whereas the shift in functional groups under Cu treatment was mainly associated with carbohydrates (C-H, COOH, etc.). The protein/polysaccharide ratios indicated that the main substances bound to Zn by *C. reinhardtii* were proteins, while polysaccharides played a greater role in the binding of Cu.

Interestingly, by comparing the removal of metals by *C. reinhardtii* before and after EPS extraction, it was found that the presence of EPS enhanced the ability of *C. reinhardtii* to accumulate Cu, while it had no significant effect on Zn (**Fig. 5**). However, to date, studies on the role of EPS in the tolerance of microalgae to toxic metal(loid) exposure have mainly focused on the dynamic secretion and surface binding characteristics of EPS after exposure (Xu and Jiang, 2015; Hou et al., 2017). Two studies determined the accumulation of As by microalgae (cyanobacteria – *Synechocystis* PCC6803 and green algae – *Chlorella vulgaris*) with and without EPS and found that they were significantly reduced after removal of EPS (Zhang et al., 2020; Naveed et al., 2020).

The combination of intracellular thiol-containing compounds (GSH, PCs) and metallothioneins (MTs) with metal ions to form organometallic complexes and transfer to vacuoles through vesicles is considered to be the preferred mode of metal detoxification in cells (Le et al., 2016; Hamed et al., 2017; Balzano et al. 2020). Metallothioneins are a type of low molecular weight protein and they can chelate metals and remove reactive oxygen species (ROS) through their thiol residues (-SH) to protect cells from oxidative damage (Le Faucheur et al., 2005; Nowicka, 2022). Phytochelatins (PCs) are synthesized through the catalysis of phytochelatin synthase (PCS) and have also been identified as main metal-binding ligands. As the precursor of PCs, glutathione (GSH) not only respond to metal ions in cells through redox reaction, but also interact with free radicals to resist oxidative stress due to its strong affinity for metals (Chen et al., 2022; Perales-Vela et al., 2006; Wesenberg et al., 2011). Therefore, GSH analysis is used to characterize the ability of metal binding in cells. With the increase in exogenous metal concentration, the main accumulation of Cu by *C. reinhardtii* changed from absorption to adsorption, and the measurement of intracellular GSH showed that Cu did not induce GSH synthesis (**Fig. 7**), which was in accordance with the complexation of this metal with EPS, resulting in an increase in metal removal and a concomitant decrease in observed toxicity. However, another study found that the intracellular GSH/GSSG ratio of *C. reinhardtii* decreased significantly with increasing Cu concentration in the culture solution, whereas the opposite trend was observed with Cd treatment (Stoiber et al., 2012). Jamers et al. (2013) assessed changes in GSH in *C. reinhardtii* under Cu exposure by flow cytometry and metabolomics and found a significant reduction in GSH levels in algal cells exposed to higher Cu concentrations. In contrast, in this study, Zn-exposed cells showed a completely opposite trend, with GSH contents of 3.0 and 11 times higher

at concentrations above 3 mg/L (**Fig. 7**). Similarly, *Dunaliella salina* showed a strong stimulatory effect of PC synthase when exposed to Zn and secreted 3~4 μmol g^{-1} PCs after 24 h of exposure (Wang et al., 2017; Tsuji et al., 2002).

In summary, the low Cu treatment (below 3 mg/L) allowed *C. reinhardtii* to grow normally through the homeostasis of Cu^{2+}, whereas at concentrations above 3 mg/L, the microalgae were subjected to intracellular oxidative stress, causing increased cell size and EPS secretion to adsorb Cu^{2+} at the cell surface. For Zn, at concentrations below 3 mg/L, Zn ions were mainly adsorbed to the binding sites on the cell wall and EPS, while at concentrations above 3 mg/L, GSH was synthesized in large quantities to scavenge H_2O_2, $O_2^{\bullet-}$ and organic radicals and transported to the vesicles to counteract Zn toxicity (Ahmad et al., 2010); however, EPS did not play a significant role in this process (**Fig. 8**).

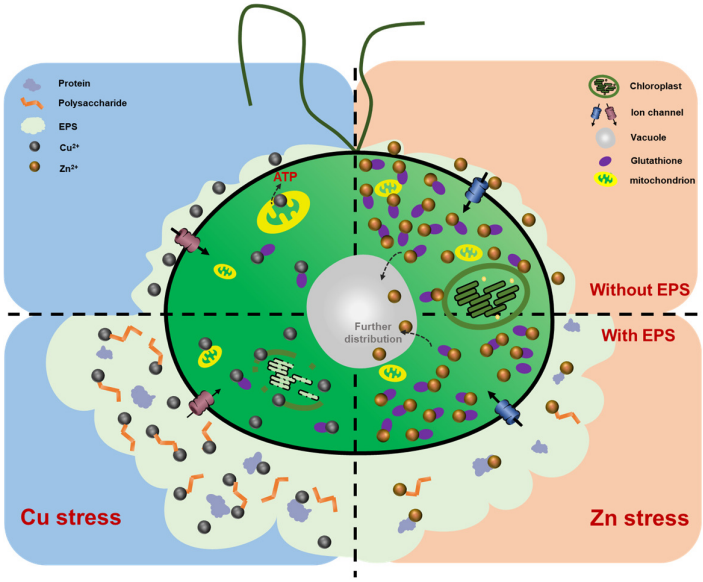

Fig. 8. Schematic diagram of the role of EPS in *C. reinhardtii* in response to Cu and Zn stress.

EPS production may be stimulated under different environmental conditions or stresses (such as light, temperature, exposure to metals and engineering nanomaterials). These macromolecules have multiple functions, including complexation of metal ions, promotion of self-flocculation, and provision of nutrients and energy to sustain growth of microbial cells (**Table S11**, Flemming and Wingender, 2010). The results in this study demonstrate that the roles of EPS in the tolerance of *C. reinhardtii* to Cu and Zn are completely different. More specifically, Cu removal may be enhanced through accelerated EPS secretion and biofilm formation. However, the detoxification of Zn may be mainly through upregulated synthesis of GSH and/or phytochelatin in cells. These findings may provide basis in the phycoremediation of aqueous environment contaminated by different metals.

5. Conclusions

In our study, the contribution of EPS and its components to Cu and Zn tolerance and intra/extracellular distribution in *C. reinhardtii* were systematically investigated. The removal of EPS significantly reduced the EC50 value of Cu, indicating that EPS strongly affected Cu toxicity thresholds as opposed to Zn. The Cu and Zn sorption patterns of *C. reinhardtii* at high and low concentrations were completely different. Moreover, multiple analyses revealed a significant

correlation of polysaccharides, proteins and DNA with Cu accumulation. Together, for the first time, we elucidated the contribution and binding ability of different components of EPS to Cu or Zn and confirmed that EPS played a minor role in the sorption and resistance of Zn by *C. reinhardtii* in our tested conditions. This study provides a new perspective for further exploring the resistance of microalgae to environmental stress by highlighting the differential role of EPS in the tolerance of *C. reinhardtii* to Cu and Zn. Further investigations may reveal strategies involving microalgae together with their EPS for the remediation of metal-contaminated sites.

CRediT authorship contribution statement

Chonghua Li: Investigation, Formal analysis, Data curation, Writing – original draft, Validation. **Peihuan Li**: Data curation, Writing – original draft. **Hongxuan Fu**: Data curation, Investigation. **Jiale Chen**: Writing – original draft, Visualization. **Menglei Ye**: Data curation, Conceptualization. **Suhua Zhai**: Data curation, Investigation. **Fan Hu**: Data curation, Visualization. **Chunhua Zhang**: Supervision, Methodology. **Ying Ge**: Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Claude Fortin**: Data curation, Methodology, Formal analysis, Writing – review & editing.

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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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161995.

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