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Nutrient removal from swine wastewater with growing microalgae at various zinc concentrations

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ABSTRACT

Coelastrella sp. grows well in swine wastewater, and could remove nutrients from swine wastewater. Some typical pollutants such as Zn(II) in swine wastewater might affect the growth of microalgae and the nutrient removal performance. In this study, *Coelastrella* sp. was cultivated in swine wastewater at various Zn(II) concentrations, and the nutrient removal capacity and physiological and biochemical properties of the microalgae were examined to better apply this technology for the removal and recovery of nutrients from swine wastewater. Results revealed that excessive Zn(II) in swine wastewater inhibited microalgae growth and reduced the increase of pH value, thus decreased ammonia nitrogen volatilization and assimilation. Remarkably in swine wastewater, Zn(II) decreased the removal efficiency of phosphorus at low Zn(II) concentration, and promoted phosphorus precipitation and stimulated assimilation of phosphorus by *Coelastrella* sp. at high Zn(II) concentration. Hence in this study, we elucidated the mechanisms of nutrient removal from swine wastewater by the microalgae *Coelastrella* sp. in the stress of Zn(II). This study could be referred in future improvement and applications of microalgae culturing in swine wastewater treatment.

1. Introduction

Microalgae culturing has been proven a cost-effective technology in wastewater treatment [1]. Microalgae-based systems have been used in the treatment of municipal [2], industrial [3], and agricultural wastewater [4]. Swine wastewater usually contains high concentrations of ammonia nitrogen (NH_3-N), phosphorus and chemical oxygen demand (COD), which poses a challenge for conventional low-cost treatment processes [5]. Compared with processes in which living organisms including duckweeds [6–8], bacteria [9–12], and wetland plants [13] were cultured for wastewater treatment, microalgae culturing for swine wastewater treatment could tolerate high concentration of NH_3-N. In addition, harvested microalgae biomass has a high content of hydrocarbons and triacylglycerol that could be converted into ethanol and biodiesel, respectively [14–17]. Therefore, microalgae culturing has a great potential in swine wastewater treatment.

Many investigations have been performed in microalgae culturing for swine wastewater treatment. Kebede-Westhead et al. [18] have reported a microalgae system for the treatment of swine manure. Raw swine manure loading rate to the system was 0.4 L m^{-2} d^{-1}; nitrogen and phosphorus in the swine manure were removed by 98.0% and 76.0%, respectively; microalgal productivity was 9.4 g dry weight m^{-2} d^{-1}. Wen et al. [19] has isolated a *Chlorella vulgaris* sp. *from* swine wastewater effluent for nutrient removal in undiluted sewage. Result has shown that the highest removal efficiencies of total phosphorus (TP) and total nitrogen were 90.51% and 91.54%, respectively after 12 days. At the same time, about 0.53 ± 0.02 g/L biomass was produced. Prandini et al. [20] have cultivated *Scenedesmus* spp. in swine wastewater for nutrient removal and biogas purification. Result has shown that growth rate of microalgae reached to 141.8 mg/L d^{-1}, and assimilation rates of NH_3-N and PO_4^{3-}-P reached 21.2 and 3.5 L $^{-1}$ d $^{-1}$, respectively. Wang et al. [21] has cultivated 4 types of green algae (*Scenedesmus obliquus*, *Hydrodictyaceae reticulatum* Lag, *Chlorella pyrenoidosa* and *Oedogonium* sp.) and 3 types of blue-green algae (*Spirulina*

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platensis, *Oscillatoria amoena* Gom and *Anabaena flos-aquae*) for nutrient removal from swine wastewater. *Oedogonium* sp. had the best performance with NH_3-N, TP and chemical oxygen demand removal rates of 95.9%, 92.9% and 62.5%, respectively. Many previous studies have investigated algal productivities and pollutant removal rates at various conditions, including nutrient loading rate [22,23], chemical oxygen demand loading rate [24], copper concentration [15], and culturing temperature [25], to optimize culturing conditions for the microalgae in swine wastewater treatment. Additionally, the C/N ratio [26], salinity [27,28] even if dissolved organic matter [29] might also influence the activity of microalgae cells. Among the reported microalgae, *Coelastrella* sp. has shown many advantages and great potential for swine wastewater treatment [15,22].

Zinc salts were often used as an additive in pig feeds for reducing disease and making pigs grow faster, but only 10–20% zinc salts were usually absorbed by pigs and the rest were excreted in swine manure and wastewater [30]. Thus, Zn(II) in swine wastewater could be one of the major concerns in many areas [31]. Previous study has reported that Zn(II) concentration could be 431–471 mg/kg in dry feces, and 2–22 mg/L in swine wastewater [32].

Zn(II) plays an important role in microalgae cell growth. For microalgae cells, Zn(II) is an important enzyme cofactor for carbonic anhydrase, superoxide dismutase (SOD), and ribonucleic acid polymerase, but high concentrations of Zn(II) might become toxic to microalgae [33], reducing the division rate, chlorophyll content, and adenosine triphosphate activity of cells in microalgae [34]. Therefore, high concentration of Zn(II) in swine wastewater might inhibit the growth of microalgae, thus affect the nutrient removal by the microalgae. In order to find the mechanism of Zn(II) stress to microalgae in nutrient removal from swine wastewater, some physiological and biochemical properties of microalgae must be study.

Chlorophyll *a* (Chl *a*) in microalgae is usually used as an indication of photosynthesis capacity. The content of Chl *a* in microalgae cells was influenced by free radicals (H_2O_2, ·OH, O_2^{2-}) which could be induced by heavy metals [35], and was also used to reflect short-term inorganic chemical toxicity in microalgae [15]. Proteins are the most important part of enzymes and organelles in microalgae cells. Responses of Chl *a* and proteins are reflecting the growth situation in microalgae cells.

Hydrogen radical (·OH) could lead to DNA damage, lipid peroxidation and protein oxidation in cells [36]. Contents of SOD and glutathione are used to assess the degree of cell repairs and environmental stress. SOD was the only enzyme to have the ability to remove O^{2-} in cells, as SOD of microalgae was able to neutralize the reactive oxygen species and catalyze intracellular O^{2-} into H_2O_2, and then preventing uncontrolled generation of ·OH [37–39]. As a water-soluble antioxidant, glutathione accumulated in plants and microalgae when their cells grow under the condition of chronic metal stress [40,41]. This process of protection and repair in microalgae cells needs consuming more energy. Adenosine triphosphate (ATP) is an energy currency molecule in cell, it releases energy when hydrolyzed, which is the direct source of energy in most organisms. In order to understand the responses and mechanisms of microalgae, specifically *Coelastrella* sp. to the stress of Zn(II) in the swine wastewater treatment, it is necessary to analyze the physiological and biochemical properties of the microalgae cells to the stress of Zn(II). SOD, glutathione and ATP are important indicator which could reflect the degree of Zn(II) stress to microalgae. This indicators could expound the exchange of nutrient removal efficiency of microalgae in Zn(II) contained swine wastewater.

In this study, we investigated the effect of Zn(II) on the performance of biomass growth and nutrient removal of the microalgae *Coelastrella* sp. in swine wastewater treatment. The responses of the microalgae reflected by physiological and biochemical properties were also studied under different concentrations of Zn(II) in swine wastewater. Critical Zn (II) concentration causing the microalgae stress was determined in the study. The mechanisms for nutrient removal by the microalgae from swine wastewater in the presence of Zn(II) were elucidated.

2. Materials and methods

2.1. Microalgae isolation and cultivation

Microalgae unialgal cultures were isolated from a piggery farm pond in Hunan, China. Homology of this microalgae reached 97% compared with *Coelastrella* sp. KU505 [22], and have been deposited in Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), Wuhan, China, and the strain number of the microalgae is FACHB-2400. Isolated microalgae strain was cultivated in 500 mL flasks with Blue-Green (BG11) medium [22]. The flasks were kept in illuminating incubator at 27 ± 1 °C, light intensity of 80 ± 5 μmol m^{-2} s^{-1}, and the daily light/dark cycle of 14:10 h. Flasks were shaken two times every day to keep light evenly radiated. Using 0.05 mol/L of Tris-HCl wash cells and adjusting pH at 7.4 by phosphate buffer saline when microalgae were inoculated at a new medium.

2.2. Swine wastewater collection

Swine wastewater was collected from a piggery farm twice at 2-month interval in Hunan, China. The fresh swine wastewater was pretreated via sedimentation and filtration. After the pretreatment, the swine wastewater was centrifugalized at 10,000 rpm for 5 min. The supernatant was collected, autoclaved at 121 °C for 30 min, and stored at 4 °C. Concentrations of NH_3-N, TP and Zn(II) in the pretreated swine wastewater were 1550 ± 15, 35 ± 3 and 2.4 ± 0.5 mg/L, respectively. The color of the swine wastewater was brownish black.

2.3. Toxicity assessment of Zn(II)

To determine the effect of Zn(II) upon microalgae growth, microalgal cells were cultivated in 500 mL flasks with 10% swine wastewater (the pretreated swine wastewater was diluted to 10% with ultrapure water). The initial biomass of microalgae cells was at OD_{680} of 0.10. $ZnSO_4$ was added to control the initial Zn(II) concentration at 0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L. The culture conditions were the same as described in above method, and microalgae were cultivated for 20 days. As the microalgae absorbed the heavy metal heavy [42], the concentration of Zn(II) in the medium was measured every 12 h and was kept constant during the whole period of culturing by adding Zn(II) solution.

2.4. Analytical methods

NH_3-N and TP were measured using the Nessler's reagent colorimetric methods and potassium persulfate digestion methods, respectively [6]. And the concentration of Zn(II) in aqueous solutions was analyzed with an atomic absorption spectroscopy (AAS, PEAA700, Waltham, MA, USA).

First-order kinetic model for NH_3-N and TP was measured by using Eq. (1):

$$C = Ae^{-rt} \quad (1)$$

The Eq. (1) was transformed to:

$$Ln(C/C_0) = LnA - rt \quad (2)$$

where *C* is NH_3-N and TP concentration (mg/L), *A* is the initial amount, *r* is the removal rate constants (day^{-1}), *t* is test time.

Morphological structure of *Coelastrella* sp. was analyzed by electron microscope (CKX41, Olympus, Japan). Microalgae dry biomass was measured using a modified method by Ho et al. [43]. A linear relationship of dry weight and optical density (OD) of microalgae *Coelastrella* sp. was reported and used in this study as Eq. (3) [15].

$$DW\ (g/L) = 0.3357 \times OD_{680},\ R^2 = 0.9962 \quad (3)$$

The biomass productivity of *Coelastrella* sp. was calculated with Eq.

(4):

$$P\ (mg/L\ day^{-1}) = (W_1 - W_0) \div (T_1 - T_0) \quad (4)$$

where W_1 and W_0 were mean dry biomass at time T_1 and T_0, respectively.

Content of Chl *a* in the microalgae biomass was determined with spectrophotometer and was calculated according to the method reported by Li et al. [44]. The content of protein was extracted from wet biomass. About 0.02 g microalgae biomass was washed by 5 mL Tris-HCl (0.05 mol/L) and put on ice, then the cells were broken using ultrasonication (XO-1000D, Nanjing Xianou Instruments Manufacture, Jiangsu, China) at 300 W for 3 min (2 s on and 5 s off). After broken, the harvested cells were centrifuged at 4000 rpm and 4 °C for 10 min, and the supernatants were dyed by Coomassie brilliant blue, and then determined with spectrophotometer [45].

Microalgae cells were pretreated according to the above method in above, the content of SOD, glutathione and ATP were measured using assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

All experiments were performed in triplicate, and the data were showed as mean ± SD (standard deviation). Data were analyzed and plotted by Origin 8.0 (OriginLab Co., USA).

3. Results and discussion

3.1. Effect of Zn(II) on nutrient removal efficiency

3.1.1. NH_3-N removal

Fig. 1 showed the changes of NH_3-N (a), TP (b), pH (c), and Zn(II) (d) in the diluted swine wastewater with various concentrations of Zn (II) during the culturing of *Coelastrella* sp. cells. As shown in Fig. 1a, the concentration of NH_3-N decreased rapidly in the first 4 days, and the decrease gradually slowed down from Day 4 to Day 20; On Day 20 when Zn(II) concentration increased from 0.50 to 2.0 mg/L, NH_3-N removal efficiency decreased from 62.3% to 39.9%. The removal efficiency of NH_3-N was 58.9%, 62.3%, 55.4%, 39.9%, 40.3%, 39.7% and 38.9% at Zn(II) concentration of 0.0, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mg/L, respectively. These results showed that NH_3-N removal efficiency changed insignificantly in the ranges Zn(II) concentration from 0.0 to 1.0 mg/L and from 2.0 to 4.0 mg/L, respectively. Therefore, compared with other factors, Zn(II) showed lesser impact on the NH_3-N removal when *Coelastrella* sp. was used to treat swine wastewater. In this study, NH_3-N was removed via microalgae cells assimilation and NH_3 volatilization, and the dominant removal mechanism was assimilation. In swine wastewater, the presence of Zn(II) could reduce the volatilization of NH_3-N, and decrease the removal ability of *Coelastrella* sp. cells.

The pH of swine wastewater was not controlled, and the variation tendency was shown in Fig. 1c. It can be seen that the pH value in the media increased with the cultivation time, and decreased with increased Zn(II) concentration. On Day 20, the highest pH was 9.52 ± 0.02 in the medium with 0.0 mg/L Zn(II), the lowest pH was 9.20 ± 0.05 in 8.0 mg/L Zn(II). When Zn(II) concentration was 0.50, 1.0, 2.0, 4.0 and 6.0 mg/L in medium, the pH was 9.44, 9.43, 9.38, 9.29 and 9.22, respectively.

The pH value in swine wastewater with different Zn(II) concentrations was shown in Fig. 1c. As shown in Figs. 2a and 1c, volatilization of ammonia was affected by pH value. NH_3-N concentration at 8.0 mg/L Zn(II) concentration (pH = 9.22) was 22.0 mg/L higher than that at 0.0 mg/L Zn(II) (pH = 9.52) on Day 20. NH_3-N concentration in swine wastewater decreased from 148 mg/L to 114.6, 120.4, 122.2, 128.6, 134.7, 136.9, 136.6 mg/L when Zn(II) concentration increased from 0.0 mg/L to 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L, respectively in 20 days. Moreover, the biomass of *Coelastrella* sp. could also affect pH

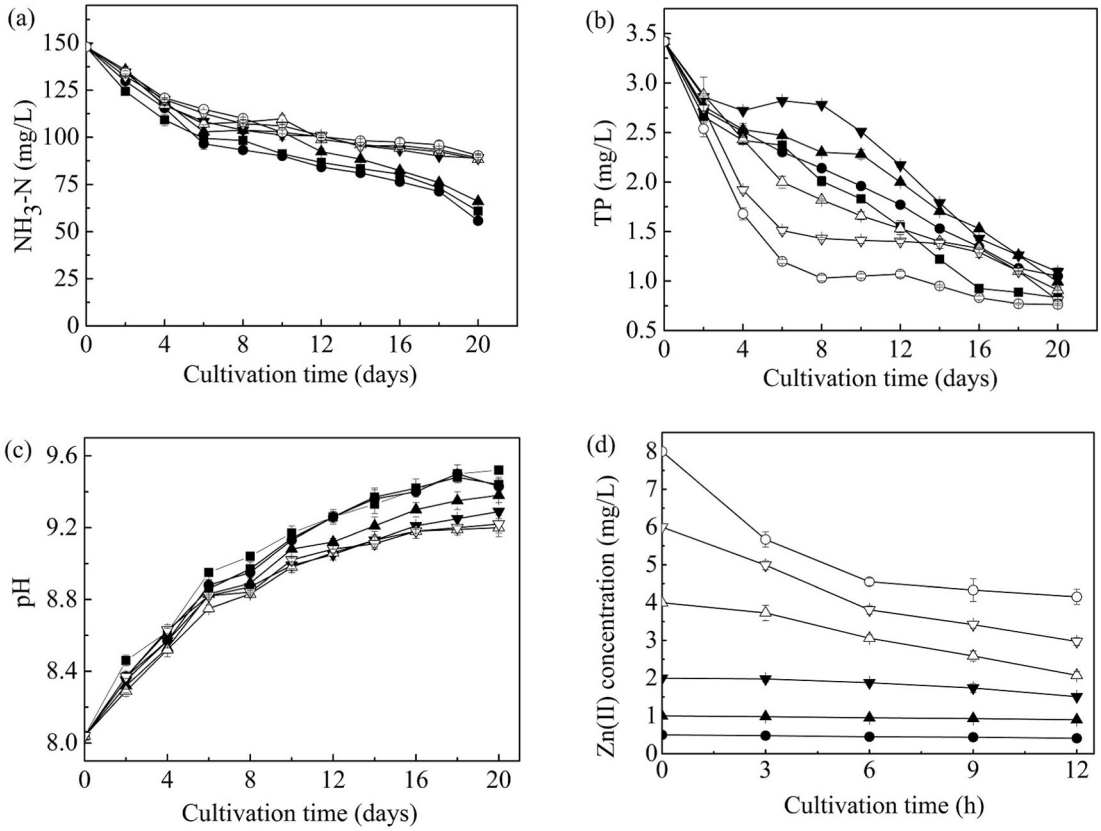

Fig. 1. Changes of NH_3-N (a), TP (b), pH (c), and Zn(II) (d) in the media with various concentrations of Zn(II) during the culturing of *Coelastrella* sp. ■: control; ●: 0.50 mg/L; ▲: 1.0 mg/L; ▼: 2.0 mg/L; △: 4.0 mg/L; ▽: 6.0 mg/L; ○: 8.0 mg/L. The data represent mean ± SD (n = 3). NH_3-N, ammonia nitrogen; TP, total phosphorus.

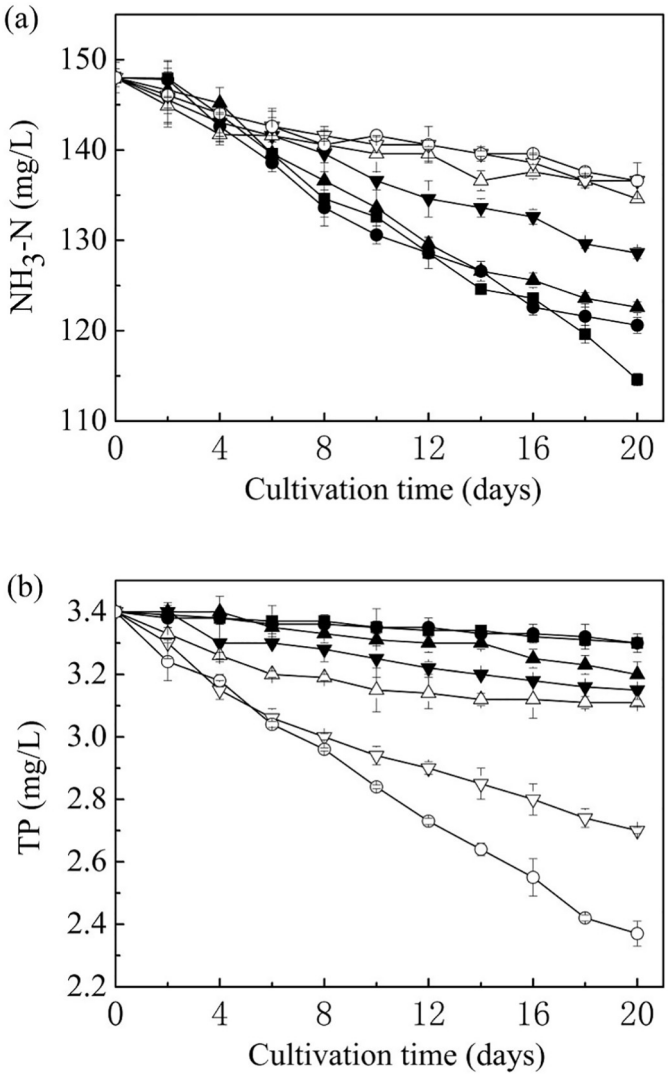

Fig. 2. NH_3-N (a) and TP (b) variation with various concentrations of Zn(II) without culturing *Coelastrella* sp. ■: control; ●: 0.50 mg/L; ▲: 1.0 mg/L; ▼: 2.0 mg/L; △: 4.0 mg/L; ▽: 6.0 mg/L; ○: 8.0 mg/L. The values represent mean ± SD (n = 3). NH_3-N, ammonia nitrogen; TP, total phosphorus.

value. The higher the biomass, the higher the pH value. The tendency for pH variation might be due to the consumed inorganic carbon by microalgae, which leaded to the homogenous release of metabolites in biodegradation of swine wastewater [46,47]. In addition, the pH value not only affected the volatilization of ammonia, but also influenced absorption of NH_3-N by *Coelastrella* sp. cells. Shimshonet al. [48] reported that pH value could influence the accumulation of ammonia of living microalgae cells, because only unprotonated ammonia could diffuse across cell membrane. When pH value increased, the uncharged species concentration increased, and ammonia could diffuse into more acidic intracellular matrix [49]. Therefore, Zn(II) could decrease the pH value indirectly, thus reduce the NH_3-N assimilation by *Coelastrella* sp. cells, and then decrease NH_3-N removal efficiency.

Fig. 2a showed NH_3-N variation with various Zn(II) concentrations without culturing *Coelastrella* sp., and the variations of pH value was controlled as same as Fig. 1c. As shown in Fig. 2a, NH_3-N concentration increased from 114.6 to 120.5, 122.6, 128.8, 134.2, 136.4 and 136.4 mg/L on Day 20, and pH value decreased from 9.52 to 9.44, 9.43, 9.38, 9.29, 9.22 and 9.20 mg/L, respectively. It suggests that the volatile quantity of NH_3-N was decreased with a decreased pH value.

3.1.2. TP removal

As shown in Fig. 1b, TP concentration decreased rapidly in all treatments at the first 4 days, and the decrease was gradually slowed down from Day 4 to Day 8 at the 6.0 and 8.0 mg/L Zn(II) concentrations. In this study, TP removal efficiency decreased with increased Zn (II) concentration when Zn(II) concentration was not higher than 2.0 mg/L. When Zn(II) concentration was higher than 2.0 mg/L, the result was opposite. The maximized removal efficiency of TP was 77.6% with 8.0 mg/L of Zn(II). When Zn(II) concentrations were 0.0, 0.50, 1.0, 2.0, 4.0 and 6.0 mg/L, the TP removal efficiency were 75.7%, 69.3%, 71.0%, 68.0%, 73.4% and 76.4%, respectively.

TP removal efficiency might be affected by the stress of Zn(II). According to a previous report, only 3–23% of phosphorus was assimilated into microalgae cells, and the rest was removed by sedimentation [14]. TP might be removed via sedimentation ($Zn_3(PO_4)_2{\cdot}2H_2O$) and the reaction route is as follows:

$$3Zn^{2+} + 4HPO4^{2-} + 2H_2O - \text{-----} Zn_3(PO_4)_2{\cdot}2H_2O + 2H_2PO_4 \quad (5)$$

$$3Zn^{2+} + 2PO4^{3-} + 2H_2O - \text{-----} Zn_3(PO_4)_2{\cdot}2H_2O \quad (6)$$

Fig. 2b showed the TP variation in various Zn(II) concentrations without cultivating microalgae. The result indicated that Zn(II) concentration could directly influence the TP concentration. With the Zn (II) concentration increased, TP concentration decreased in all the tests, especially when Zn(II) concentration was higher than 4.0 mg/L. TP concentration decreased from 3.4 mg/L to 3.3, 3.3, 3.2, 3.15, 3.11, 2.7 and 2.37 mg/L in 0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L Zn(II) media, respectively. This result showed that a part of TP in swine wastewater was combined with Zn(II) to precipitate, and the amount of precipitation increased with the increase of Zn(II) concentration. In brief, the increased of Zn(II) concentration could improve TP precipitation in swine wastewater.

The data concerning the variations of Zn(II) concentration of the medium in 12 h was shown in Fig. 1d. The result showed that Zn(II) concentration decreased to 4.2, 2.9 and 2.1 mg/L when the initial concentration were 8.0, 6.0 and 4.0 mg/L, respectively in swine wastewater. And has an inconspicuous decreased when initial Zn(II) concentration was not higher than 2.0 mg/L.

Fig. 2b showed that TP concentration decreased significantly at the Zn(II) concentrations were 6.0 and 8.0 mg/L within 20 days, and decreased slowly with an decreased Zn(II) concentration. These results were consistent with the data of TP removal efficiency in Fig. 1d. The results indicated that Zn(II) concentration might enhance the removal efficiency of TP via sedimentation, especially in high Zn(II) concentrations. On Day 20, quantitative analyses of TP removal efficiency at different routes were performed (Table 1). TP removal efficiency decreased with an increased of Zn(II) when TP was removed via

Table 1
TP removal efficiency of different route in various concentration of Zn(II) (on the Day 20).

Removal route of TP	TP concentration in various concentration of Zn(II) (mg/L)						
	0.0	0.5	1.0	2.0	4.0	6.0	8.0
Total removal (mg/L)	2.59	2.37	2.43	2.33	2.33	2.61	2.66
Sedimentation (mg/L)	0.1	0.1	0.2	0.25	0.29	0.7	1.03
Assimilation (mg/L)	2.49	2.27	2.23	2.08	2.04	1.91	1.63

Table 2
Important parameters and statistics indexes of the first-order kinetic model for NH_3-N and TP removal from swine wastewater at various Zn(II) concentrations. Data were expressed as mean ± SD (n = 3).

Zn (mg/L)	NH_3-N		TP	
	Removal rate constants (r_f)	Initial adsorption amount	Removal rate constants (r_f)	Initial adsorption amount
0.0	0.0362 ± 0.0004	0.1004 ± 0.0007	0.0725 ± 0.0024	0.0037 ± 0.0001
0.50	0.0398 ± 0.0006	0.0568 ± 0.0007	0.0559 ± 0.0015	0.0488 ± 0.0013
1.0	0.0358 ± 0.0005	0.0453 ± 0.0007	0.0534 ± 0.0005	0.0049 ± 0.0006
2.0	0.0258 ± 0.0004	0.0488 ± 0.0011	0.0533 ± 0.0002	0.0166 ± 0.0009
4.0	0.0257 ± 0.0003	0.0376 ± 0.0005	0.0599 ± 0.0008	0.0837 ± 0.0007
6.0	0.0248 ± 0.0009	0.0685 ± 0.0013	0.0642 ± 0.0014	0.0580 ± 0.0015
8.0	0.0221 ± 0.0001	0.0837 ± 0.0005	0.0770 ± 0.0034	0.1388 ± 0.0025

assimilation. These might because biomass of *Coelastrella* sp. decreased with an increased Zn(II) concentration and with culturing duration. As shown in Fig. 1b, TP removal efficiency at 4.0, 6.0 and 8.0 mg/L Zn(II) concentration was higher than 0.50, 1.0 and 2.0 mg/L Zn(II) on the whole, however, most TP were removed via sedimentation. Excess Zn (II) could inhibited the growth of *Coelastrella* sp. and then decreased the assimilation of TP.

3.1.3. First-order kinetic model

The results of the NH_3-N and TP removal at various Zn(II) concentrations fit well with the first-order kinetic model. The important parameters of the first-order kinetic model are shown in Table 2. The r_f is the removal rate constants, the highest r_f of NH_3-N was 0.0398 ± 0.0006 at the 0.50 mg/L Zn(II) concentration in swine wastewater, and with Zn(II) concentrations increased from 0.50 to 8.0 mg/L, r_f of NH_3-N was decreased from 0.0398 to 0.0221. The lowest r_f of TP has found in 2.0 mg/L, it was around 0.0533 ± 0.0002. When Zn(II) concentrations increased from 0.0 to 2.0 mg/L, r_f of TP was decreased from 0.0725 to 0.0533, and with Zn(II) concentrations increased from 2.0 to 8.0 mg/L, it was increased from 0.0533 to 0.0770. This result was in accordance with the result of Fig. 1a and b.

3.2. Effect of Zn(II) on microalgae biomass

Fig. 3 showed the dynamic changes of microalgae *Coelastrella* sp. biomass at various concentrations of Zn(II) in swine wastewater. Biomass increased rapidly at the first 2 days in all treatments, and the growth rate decreased with an increased Zn(II) concentration. After

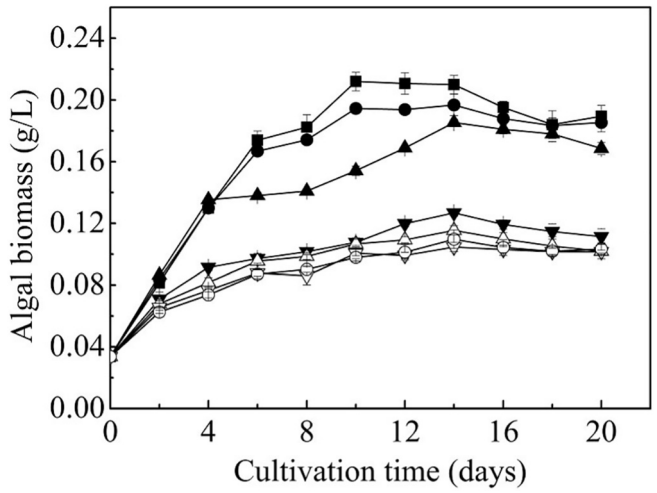

Fig. 3. Dynamic changes of *Coelastrella* sp. biomass with various concentrations of Zn(II) in swine wastewater. ■: control; ●: 0.50 mg/L; ▲: 1.0 mg/L; ▼: 2.0 mg/L; △: 4.0 mg/L; ▽: 6.0 mg/L; ○: 8.0 mg/L. Data were expressed the average and standard deviation in biological duplicates (n = 3).

2 days, the growth rate of *Coelastrella* sp. became low when the concentration of Zn(II) was not higher than 2.0 mg/L. The biomass of *Coelastrella* sp. stopped increasing and dropped gradually on Day 12–14. And biomass of *Coelastrella* sp. decreased from 0.190 to 0.103 g/L when the Zn(II) concentration increased from 0.0 to 8.0 mg/L on Day 20. Biomass productivity was 7.79, 7.58, 6.75, 3.89, 3.41, 3.39 and 3.48 mg/L day^{-1} at 0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L Zn(II) concentration, respectively. When Zn(II) concentration in media was higher than 2.0 mg/L, the biomass of *Coelastrella* sp. decreased to 58.8% of the control. This result was in accordance with the result of Omar et al. [34], In their study, the authors cultivated *S. obliquus* and *S. quadricauda* at various concentrations of Zn(II) and reported that Zn(II) promoted the growth of the microalgae at low concentrations, but about 24%–33% of growth inhibition appeared when microalgae cells were exposed to high concentration of Zn(II). Monteiro et al. [33] has reported the growth of *D. pleiomorphus* and *S.obliquus* in medium under stress of Zn(II). Results showed the inhibition rates of microalgae cells were 15–20% with 2.0 mg/L Zn(II) concentration, and 37–60% with 8.0 mg/L Zn(II). These results were in agreement with ours study. Zn(II) might reduce the growth of *Coelastrella* sp. cells and affect its physiological and biochemical properties, which could influence the removal efficiency of NH_3-N and TP indirectly.

Fig. 4 showed the electron microscope images of *Coelastrella* sp. with various Zn(II) concentration in swine wastewater on Day 20. In this study, image of *Coelastrella* sp. in 0.0 and 0.5 mg/L Zn(II) has no significant difference. The distribution of *Coelastrella* sp. was dispersive in 0.0 and 1.0 mg/L Zn(II) concentration (Fig. 4a and b), and the shape of *Coelastrella* sp. cells was more full and regular. When Zn(II) concentration increased from 1.0 to 4.0 mg/L, *Coelastrella* sp. cells began to agglomerate, and the shape of cells became more flat and irregular (Fig. 4d, e and f). Excess Zn(II) damaged structure of *Coelastrella* sp. cells, and also enhanced agglomeration among single cells.

Figs. 1 and 3 showed that NH_3-N and TP concentrations decreased when microalgae biomass increased. Nitrogen is an important element in microalgae cells and is involved in the syntheses of proteins, enzymes, chlorophylls and genetic materials, and microalgae cells tend to assimilate ammonia as the nitrogen source than other N species [22]. Phosphorus plays a vital role in microalgae cell growth, especially to the generation and metabolism of energy. Phosphorus also influences the biomass, cell division and synthesis of chlorophyll in microalgae [49–51]. Zn(II) could inhibited the growth of microalgae, thus decreased the removal of NH_3-N and TP.

3.3. Effect of Zn(II) on chlorophyll a and protein

As a common indicator of photosynthesis, chlorophyll *a* (Chl *a*) reflects the growth of microalgae. Content of Chl *a* was measured to determine the damage caused by Zn(II) in this study. Fig. 5a showed the content of Chl *a* of *Coelastrella* sp. cells in all treatments (mg Chl/g biomass). The maximum content of Chl *a* was found at 1.0 mg/L of Zn (II), correspondingly to 1.06 ± 0.02 mg/g in *Coelastrella* sp. cells. And

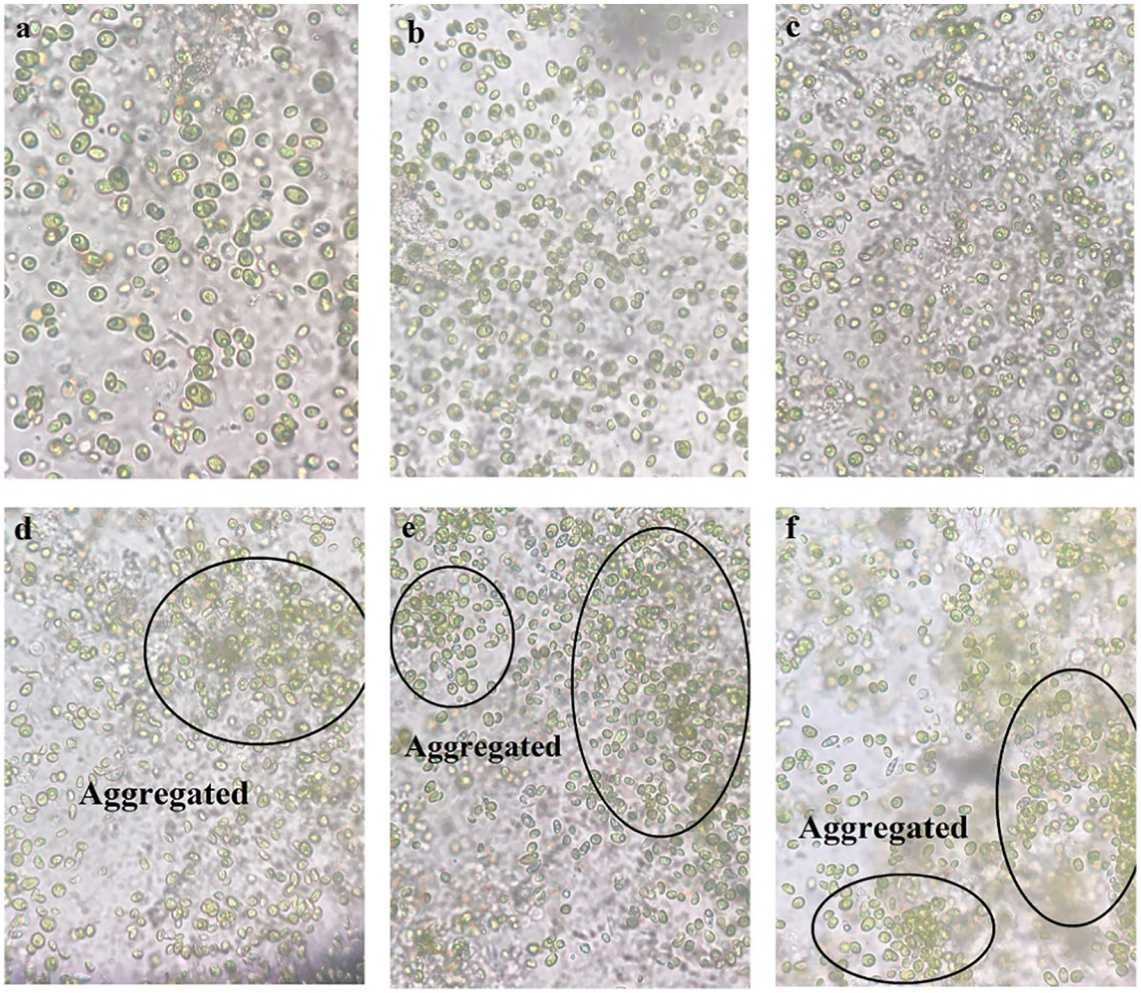

Fig. 4. Electron microscope image of *Coelastrella* sp. with various concentration of Zn(II) in swine wastewater on Day 20. (a: 0.0 mg/L, b: 1.0 mg/L, c:2.0 mg/L, d: 4.0 mg/L, e: 6.0 mg/L, f: 8.0 mg/L).

the content of Chl *a* was 0.85, 0.97, 0.98, 0.75, 0.66 and 0.59 mg/g when Zn(II) concentration was 0.0, 0.50, 2.0, 4.0, 6.0, 8.0 mg/L, respectively. This result showed that low Zn(II) concentration (not higher than 1.0 mg/L in this study) permitted a gradual increase of Chl *a* in *Coelastrella* sp. cells. On the contrary, when the concentration of Zn(II) increased from 1.0 to 8.0 mg/L, the content of Chl *a* decreased from 1.06 to 0.60 mg/L. Cao et al. [52] found the same results working with green algae *Cladophora*. Chl *a* played an important role in *Coelastrella* sp. cells growth. In this study, when Zn(II) concentration was higher than 1.0 mg/L, the effects of Zn(II) on Chl *a* showed that the higher Zn (II) concentration, the lower Chl *a* content in cells (Fig. 5a). Previous study has reported that Zn(II) is an essential element in Chl *a* of microalgae cells [46]. However, excess of Zn(II) might blind the sulphydryl groups (-SH), which played a significant role in catalytic action and structural integrity of enzymes in microalgae cells [53], and the inhibition of enzyme hindered the synthesis of Chl *a*. Küpper et al. [54] found that Zn(II) could replace Fe(II) and Mg(II) in protein SH groups, reduce the content of Chl *a* and damage the photosynthesis in cells. As shown in Fig. 3 and Table 1, biomass of *Coelastrella* sp. decreased significantly when Zn(II) concentration increased from 1.0 to 2.0 mg/L. These results was consistent with the change of Chl *a*. When the concentration of Zn(II) was higher than 1.0 mg/L, Zn(II) might damage Chl *a* in cells and reduced the biomass of *Coelastrella* sp., and consequently led to a drop in the NH_3-N removal efficiency by *Coelastrella* sp. cells. Therefore, Zn(II) reduced NH_3-N removal capacity of *Coelastrella* sp. by reduced the growth of the microalgae cells. So, the higher the biomass mass, the higher the NH_3-N removal efficiency in swine wastewater.

Proteins are a very important component for microalgae cells. As shown in Fig. 5b, protein content showed a strong positive correlation with Zn concentration. The intracellular protein content of *Coelastrella* sp. increased with an increased Zn(II) concentration. When *Coelastrella* sp. was cultured with 0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L of Zn(II), the content of protein in *Coelastrella* sp. cells (g protein/g biomass) were 0.097, 0.090, 0.102, 0.149, 0.193, 0.205 and 0.207 g/g, respectively. This result was in agreement with the study by Malea et al. [55]. When exposed to Zn(II), *Coelastrella* sp. cells might produce more protein against toxicity of Zn(II). As shown in Fig. 5b, the higher the Zn(II) concentration, the higher the protein concentration. The result might attribute to the synthesis of anti-oxidative, Zn-binding proteins, and peptide-disulfide, leading to a "Zn–protein interaction" in cells [56]. The proteins could reduce the metal toxicity through inducing the synthesis of protective proteins in microalgae cells [57]. The increase of proteins in microalgae also might be due to the increased phytochelatins, which involved in cellular detoxification through formative metal complexes [58].

3.4. Effect of Zn(II) on SOD, glutathione and ATP

The content of SOD, glutathione and ATP in *Coelastrella* sp. cells were analyzed at the microalgae growing period, in which had a high activity of microalgae cells under the stress of Zn(II). Therefore in this study, the content of SOD, glutathione and ATP in *Coelastrella* sp. cells

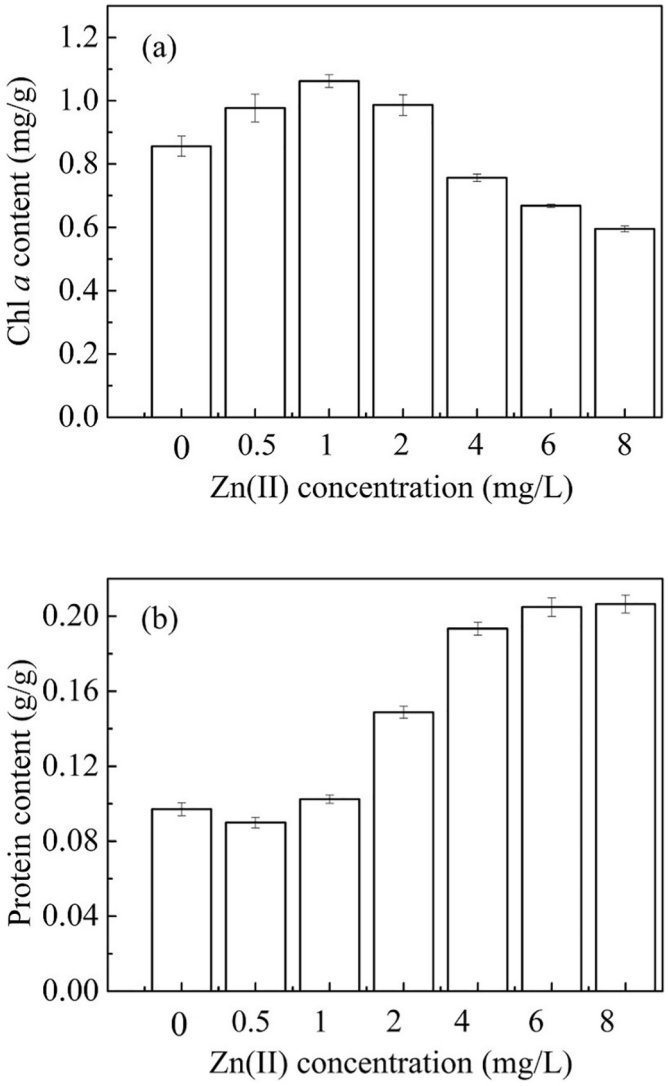

Fig. 5. Content of chlorophyll *a* (a) and protein (b) in *Coelastrella* sp. with various concentrations of Zn(II) in swine wastewater on Day 20. Data were expressed the average and standard deviation of three biological replicates.

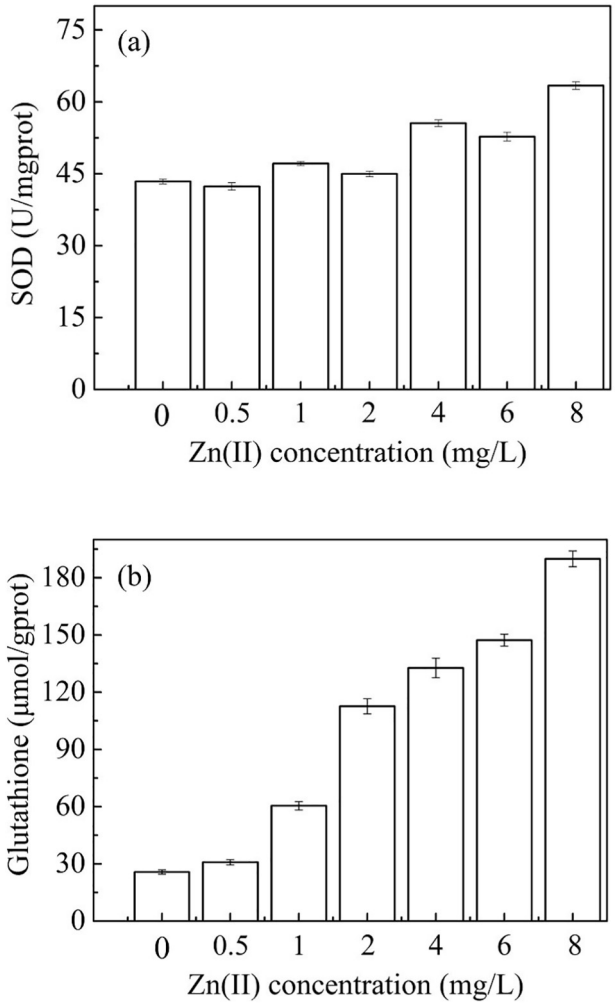

Fig. 6. Content of SOD (a) and glutathione (b) in *Coelastrella* sp. with various concentrations of Zn(II) in swine wastewater on Day 8. Data were expressed the average and standard deviation in biological duplicates (n = 3). SOD, superoxide dismutase.

were measured on Day 8.

As an enzyme involved in the removal of oxygen free radical in microalgae cells, the activity of SOD was measured when microalgae cells were exposed to Zn(II). Fig. 6a showed the activity of SOD in *Coelastrella* sp. cells on Day 8. When Zn(II) concentration increased, the content of SOD in *Coelastrella* sp. cells increased gradually. The maximum content of SOD was around 63.4 U/mg prot with 8.0 mg/L of Zn (II), and the content of SOD was 43.4, 42.3, 47.1, 44.9, 55.5 and 52.7 U/mg prot, respectively. These results are in accordance with those reported by Hamed et al. [59] and Li et al. [15]. The response of *Coelastrella* sp. to Zn(II) stree showed that high concentration Zn(II) could stimulate absorption of TP by microalgae. As shown in Fig. 6a, content of SOD increased with an increased Zn(II) concentration, and maximized at 8.0 mg/L Zn(II) concentration, which was corresponding to the highest TP removal efficiency. When microalgae were exposed to high Zn(II) concentration, the microalgae generated more reactive oxygen species (ROS), which could lead to damage in algae including photosynthetic reduction and viability loss [60]. To prevent and counteract such damage by ROS, microalgae should adjust the protective/harvesting pigment ratio for regulating energy flow towards the photo systems [61]. When the prevention mechanisms could not adjust intracellular environment, superoxide (O_2^-) is formed, and SOD is capable of O_2^- removal. So, the concentration of SOD could reflect the degree of stress. In this study, when Zn(II) concentration was not lower than 1.0 mg/L, content of SOD in *Coelastrella* sp. increased slightly, suggesting that there were some prevention mechanisms in *Coelastrella* sp. cells and the degree of prevention increased with the increase of Zn (II) concentration.

Glutathione is an antioxidant in microalgae cells. It can protect unstable macromolecules, clear free radicals and hydrogen peroxide, and sever toxic oxidation derivatives to maintain the activity of photosynthesis [62]. Content of Glutathione was another parameter which played a role of the metal toxicity indicator in microalgae cells [55]. As shown in Fig. 6b, the effect of Zn(II) on glutathione in *Coelastrella* sp. cells was not obvious when the concentration of Zn(II) was lower than 0.50 mg/L. Once the concentration of Zn(II) increased to 1.0 mg/L, the content of glutathione increased sharply. Content of glutathione increased with an increased Zn(II) concentration, and the content of glutathione in *Coelastrella* sp. cells were 25.7, 30.8, 60.4, 112.6, 132.7, 147.3, and 189.9 mg/g with corresponding Zn(II) concentration of 0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg/L, respectively. Content of glutathione in the *Coelastrella* sp. cells under 8.0 mg/L Zn(II) concentration was about 7.4 times higher than that of the control group. This result was supported by Hamed et al. [59], who cultivated *Chlorella sorokiniana* and *Scenedesmus acuminatus* under Zn(II) stress. The glutathione is a metal ligand in cells and can protect against pro-oxidants

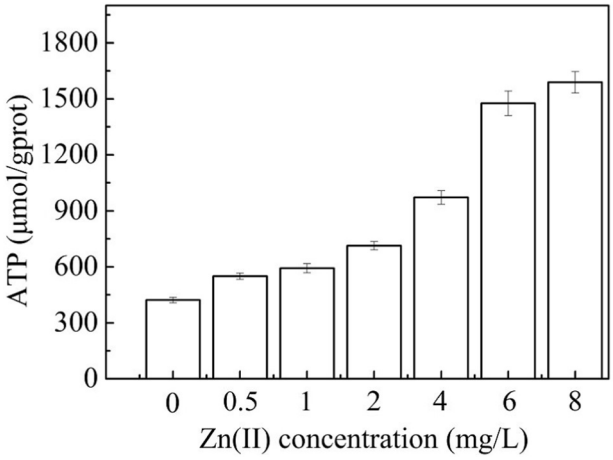

Fig. 7. Content of ATP in *Coelastrella* sp. with various concentrations of Zn(II) in swine wastewater on Day 8. Data were expressed the average and standard deviation in biological duplicates ($n = 3$). ATP, adenosine triphosphate.

through formation of complexes with metals. When under metals stress, interaction of metals with glutathione metabolism might be a part of the toxic response of metals [63]. As shown in Fig. 6b, glutathione content of *Coelastrella* sp. increased obviously when Zn(II) concentration increased from 0.50 to 1.0 mg/L. As shown in Fig. 6a and b, *Coelastrella* sp. produced more glutathione than SOD in defense against Zn(II). When exposed to Zn(II), *Coelastrella* sp. produced glutathione which combined with Zn(II) and the reaction between Zn(II) and glutathione might as follows:

$$4GSH + 2Zn^{2+} - -----2Zn^{+} - SG + GSSG + 4H^{+} \quad (7)$$

where GSH is glutathione, GSSG is glutathione disulfide.

At Zn(II) concentration lower than 0.50 mg/L, *Coelastrella* sp. could adjust intracellular environment via the combination of glutathione with Zn(II). The toxic Zn(II) did not induce ROS, so the SOD content increased slightly with the increase of Zn(II) concentration. When Zn(II) concentration increased from 1.0 to 8.0 mg/L, SOD content of *Coelastrella* sp. increased slowly, while glutathione increased significantly. This result suggested that glutathione was the main substance against Zn(II) concentration in *Coelastrella* sp. cells. When Zn(II) concentration was higher than glutathione handling capacity, it could induce ROS, and then SOD content increased.

ATP is the energy currency of microalgae cells, and many studies have researched microalgae by analyzing ATP. However, the investigation of ATP content in microalgae under Zn(II) stress in treatment of swine wastewater has not been reported in the literature. Fig. 7 shows that the content of ATP increased with an increased Zn(II) concentration. In this study, ATP content increased significantly when Zn (II) concentration increased to over 4.0 mg/L, and the maximum content of ATP was 1589 ± 57 μmol/g prot at 8.0 mg/L of Zn(II). Combined with Figs. 1b and 2, it can be seen that less biomass of *Coelastrella* sp. was observed in the experimental groups containing 4.0, 6.0 and 8.0 mg/L of Zn(II), but the removal efficiency of TP was higher than that in all other experimental groups. All of the biological preservation behaviors of *Coelastrella* sp. cells need to consume energy. Fig. 7 showed that ATP content increased slightly at the Zn(II) concentration of 0.50, 1.0 and 2.0 mg/L, and increased obviously when Zn(II) concentration was higher than 4.0 mg/L. This result was corresponding to glutathione content in Fig. 6b, and suggesting that once *Coelastrella* sp. cells exposed to Zn(II), cells produced glutathione against toxicity of Zn (II). Therefore this process consumed ATP, and then increased the TP removal efficiency in swine wastewater. ATP in algal cells could regulate the balance of intracellular environment through chelating heavy metals [64]. Moreover, some energy must be assigned to maintain the antioxidant capacity of cells when under environmental stress [65]. This result showed that high Zn(II) concentration induced high synthesis of ATP, and more TP was consumed in swine wastewater.

4. Conclusions

NH_3-N and TP in swine wastewater could be removed with growing microalgae in the presence of Zn(II). NH_3-N removal efficiency maximized 62.3% at 0.50 mg/L of Zn(II), and maximal TP removal was 77.6% at 8.0 mg/L of Zn(II). When Zn(II) concentration was increased, the growth rate of *Coelastrella* sp. decreased, and the content of proteins, SOD, glutathione and ATP increased. High Zn(II) concentration could inhibit the growth of *Coelastrella* sp., thus slow down the increase of pH, and ultimately reduced NH_3-N volatilization and assimilation. Zn (II) also enhanced TP precipitation and phosphorus assimilation via *Coelastrella* sp. in swine wastewater.

Authors' agreement to authorship and submission

All persons designated as author agree to submit the manuscript for peer review.

Declaration of competing interest

The authors declare there no conflict of interest that could perceive to influence the results of the research.

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Declaration of authors' contribution

Xiang Li designed the study and wrote the paper, Shaohua Wu, Yan Lin and Yuanyuan Zhong conducted most of the experiments, Qi Zhou, Lijun Nie and Cheng Du analyzed and interpreted the data in this study, Chunping Yang, Guangming Zeng and Wei Lou designed the study, and Chunping Yang and Guangming Zeng revised the article critically.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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