Effects of copper and zinc on pollutants removal in horizontal subsurface flow constructed wetlands

Yan Zhang[a,b], Hongyun Dong[a,b], Xinhua Li[a,b,*], Piet N.L. Lens[c], Nana Wang[a,b], Hongyuan Liu[a,b], Yanjun Wang[a,b], Ying Li[a,b]

[a]*Shandong Engineering Laboratory of Wetland and Eco-Agriculture, Institute of Wetland Agriculture and Ecology, Shandong Academy of Agricultural Sciences, Ji'nan 250100, P.R. China, emails: xinhuali_2008@163.com (X. Li), swallow928@126.com (Y. Zhang), donghy0102@163.com (H. Dong), wangnana269@126.com (N. Wang), saasliuhongyuan@163.com (H. Liu), wang.yanjun1985@163.com (Y. Wang), liyingsysu@foxmail.com (Y. Li)*
[b]*Institute of Modern Agriculture on Yellow River Delta, Shandong Academy of Agricultural Sciences, Ji'nan 250100, P.R. China*
[c]*National University of Ireland Galway, University Road, H91 TK33, Galway, Ireland, email: piet.lens@nuigalway.ie (P.N.L. Lens)*

ABSTRACT

A series of horizontal subsurface flow constructed wetlands with *Phragmites australis* were set up to evaluate the removal efficiency of nitrogen, phosphorus and chemical oxygen demand (COD) under different influent copper (Cu^{2+}) or zinc (Zn^{2+}) concentrations, and they were operated intermittently with a 9-d residence time (RT). Different Cu^{2+} and Zn^{2+} (0, 2, 4, 8, and 16.32 mg/L) levels were set up in the synthetic influent water prepared in eleven feed tanks, respectively. With an increase of Cu^{2+} concentrations, the removal efficiency of total nitrogen (TN) and ammonium nitrogen (NH_4^+–N) increased, and the optimal Cu^{2+} concentration was 2 mg/L to enhance nitrogen removal. The presence of Cu^{2+} promoted the bioactivity of anaerobic ammonia-oxidizing bacteria bioactivity. Because of Zn^{2+} complexed by recalcitrant fractions of organic matter, the presence of significantly inhibited TN and nitrate nitrogen (NO_3^-–N) removal, while Zn^{2+} slightly improved COD removal in the constructed wetlands. Both of Cu^{2+} and Zn^{2+} did not affect total phosphorus removal in constructed wetlands. The presence of Zn^{2+} may promote plant metabolism and an ability of roots to secrete oxygen, which increased oxidation–reduction potential and affected denitrification in constructed wetlands.

Keywords: Constructed wetlands; Nitrogen and phosphorus removal; Organic substances; Cu^{2+}; Zn^{2+}
Phragmites australis

1. Introduction

Horizontal subsurface flow constructed wetlands (HSSF-CWs) can be built and managed as low cost, small floor area, relatively simple operation and maintenance, as well as little secondary pollution; they exhibit high removal efficiency of organic matter, nutrients, microplastics, and heavy metals from wastewater [1–3]. Heavy metals, especially copper (Cu) and zinc (Zn) are widely present in livestock and poultry breeding wastewater [4,5]. Cu and Zn in the wastewater are inevitable, since the bioavailability of Cu and Zn is only 5%–30% of livestock and poultry feed because of low utilization rates by animals, and 70%–95% of Cu and Zn are mainly excreted with animal feces [6]. However, Cu and Zn have been extensively used as feeding additives to increase production and improve the economic income of enterprises in the intensive and large-scale livestock and poultry feeding industry at present [7–9], which lead to the phenomenon that more Cu and Zn are retained in animal feces, and then enter into the water body through leaching and direct

* Corresponding author.

runoff, thereby causing heavy metal contamination [10]. For instance, Cu and Zn concentrations were 1.92–9.81 mg/L and 1.30–9.25 mg/L, respectively, in the wastewater from livestock and poultry farms in China [11–13], which indicates that there is serious Cu and Zn pollution in livestock and poultry breeding wastewater. Meanwhile, livestock and poultry breeding wastewater contains high nitrogen, phosphorus and organic matter concentrations [14]. Hence, HSSF-CWs could play a vital role in treating the livestock and poultry breeding wastewater.

Cu is an essential micronutrient required for plant growth and development, since Cu are associated with proteins and enzymes involved in electron transfer and redox reactions in plants [15,16]. However, excess Cu can disturb and inactivate protein structure as a result of unavoidable binding to proteins, which can interfere with photosynthesis, pigment synthesis, nitrogen and protein metabolism, membrane integrity and mineral uptake, and thereby excess Cu could affect the ability of HSSF-CWs to remove contaminants. As an essential trace element for plant growth, Zn is an important component in the synthesis of carbonic anhydrase affecting plant photosynthesis, and a component of many enzymes of cellular biological processes including protein metabolism, gene expression, and chromosome construction [17–19], indicating that Zn can also promote plant growth and development. However, excessive Zn not only reduces the metabolic activity of plant rhizosphere microorganisms and plant growth and development [20,21], but also inhibit the anammox activity [22]. Meanwhile, the presence of excess Cu and Zn in wastewater can also inhibit the microbial activity and plant growth in constructed wetlands (CWs), affecting the nitrification and denitrification processes, and consequently limit the purification efficiency of CWs [23–25]. However, previous studies provided limited information to the contrasting influence of Cu and Zn on the amount of different nitrogen species, phosphorus and chemical oxygen demand (COD) by CWs simultaneously. There was also little information on the change

of oxidation–reduction potential (ORP) in CWs upon Cu and Zn exposure. Therefore, the comprehensive and more detail-oriented research is necessary for better understanding of the impact of Cu and Zn on the wastewater treatment efficiency in CWs, especially on the simultaneous removal of nitrogen, phosphorus and carbon.

The purpose of this study was to (1) examine the effect of cupric (Cu^{2+}) or zinc (Zn^{2+}) ions on removal contaminants from wastewater (2) evaluate the capacity of HSSF-CWs to remove contaminants with the presence of Cu^{2+} and Zn^{2+}, and (3) reveal the effect of Cu^{2+} and Zn^{2+} on the ORP and pH of HSSF-CWs. The study will provide a new insight on the mechanisms of contaminant in livestock and poultry breeding wastewater, and valuable information for contaminant removal from livestock and poultry wastewater, and also broaden the application scope of CWs to various types of wastewater treatment.

2. Material and methods

2.1. Experimental design operation

The experiment was established and operated at a test site of Shandong Academy of Agricultural Science (Ji'nan, China). The mean temperature during the experiment was 25–34°C. Identical PVC (12 mm thick, W0-W10) horizontal subsurface flow microcosm constructed wetlands were set up with the size of: 2.0 m length, 0.8 m width, 0.95 m height (Fig. 1). Each wetland bed was filled with a 0.75-m layer of large slag (5–30 mm in diameter) at the bottom and a 0.1 m layer of small slag (<5 mm in diameter) and soil on the top. Each wetland included the inlet section (0.25 m long) with gravel (20–30 mm), working section (1.5 m long), and outlet section (0.25 m long) with gravel (20–30 mm). *Phragmites australis* was selected due to its large biomass and root zone as well as effective pollutant removal performance, and had a planting density of 45 plants/m². Plants and bacterial communities in wetlands were cultivated mainly using 1/20
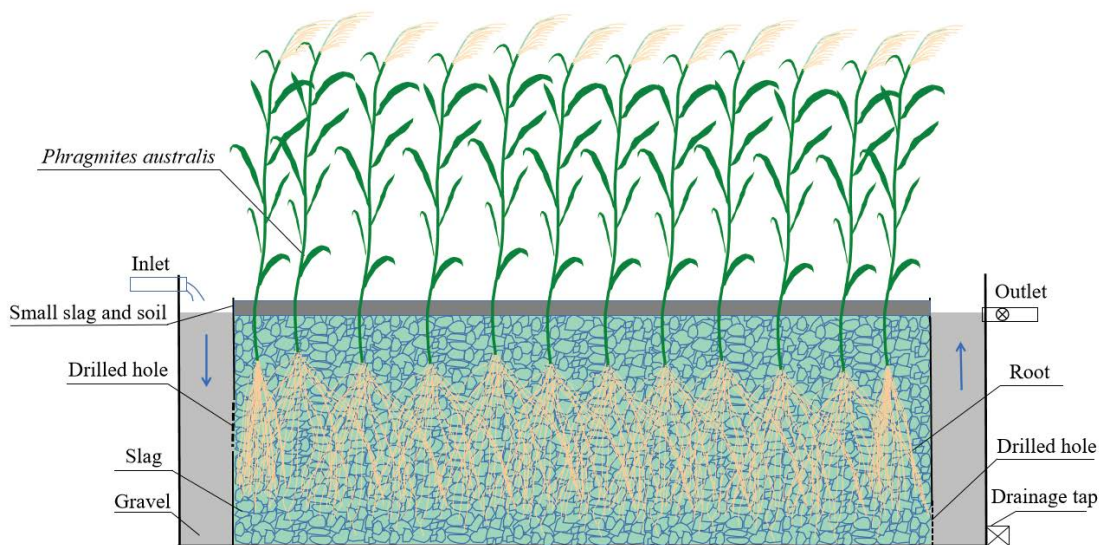

Fig. 1. Schematic diagram of the experimental HSSF-CWs used in this study.

Hoagland modified nutrient salts solution for four months main to the experiment.

Synthetic influent was prepared in eleven feed tanks by dissolving ammonium nitrate, glucose, sodium dihydrogen phosphate with tap water for the sake of minimizing the fluctuation of influent characteristics and comparing the results of parallel experiments. The total nitrogen (TN), total phosphorus (TP) and COD concentrations in synthetic influent were modified to 210, 15 and 490 mg/L in all treatments. The wetland without addition of Cu or Zn was W0. Following the addition of the $CuSO_4·5H_2O$ was adjusted Cu^{2+} concentrations (2, 4, 8, 16 and 32 mg/L) in the synthetic wastewater for wetlands (W1, W2, W3, W4, W5), while the addition of the $ZnSO_4·7H_2O$ was adjusted Zn^{2+} concentrations (2, 4, 8, 16 and 32 mg/L) for other wetlands (W6, W7, W8, W9, W10). The error range of the design concentrations and actual concentrations were all within 5.0% for assuring the effectiveness of designed experiments. Reactors were operated intermittently with a 9-day residence time (RT) in the batch experiment. The synthetic influent water was introduced into the reactors at the same time. Three replicated microcosms were set up for each respective influent Cu^{2+} or Zn^{2+} concentrations.

2.2. Sample collection

Effluent water samples collected from the outlet sections were used to measure ammonium nitrogen (NH_4^+–N), nitrate nitrogen (NO_3^-–N), TN, TP, COD, Cu^{2+}, Zn^{2+}, pH, and ORP. Cu^{2+} and Zn^{2+} concentrations were analyzed via an atomic absorption spectrophotometry (Persee A3, Beijing, China). ORP and pH were measured with a Sension ORP electrode (PHBJ-261L, Shanghai, China) and Piccolo pH meter (Mettler Toledo S210, Shanghai, China), respectively. COD was measured via the potassium dichromate method as outlined by Zhang et al. [26]. Other parameters were analyzed using an ultraviolet-visible (UV-Vis) spectrophotometer (UV-8000S, METASH, Shanghai, China) according to APHA Methods (1998).

2.3. Statistical analysis

The SPSS 16.0 statistical package was used for statistical analysis, and the removal efficiencies and contents of contaminants were performed with two-way analysis of variance (ANOVA). All data are presented as the standard deviation (SD) of three replicates. Origin (v.8.5 Origin Lab) was used to plot water quality figures.

3. Results and discussion

3.1. Nitrogen removal under various influent Cu^{2+} and Zn^{2+} concentrations

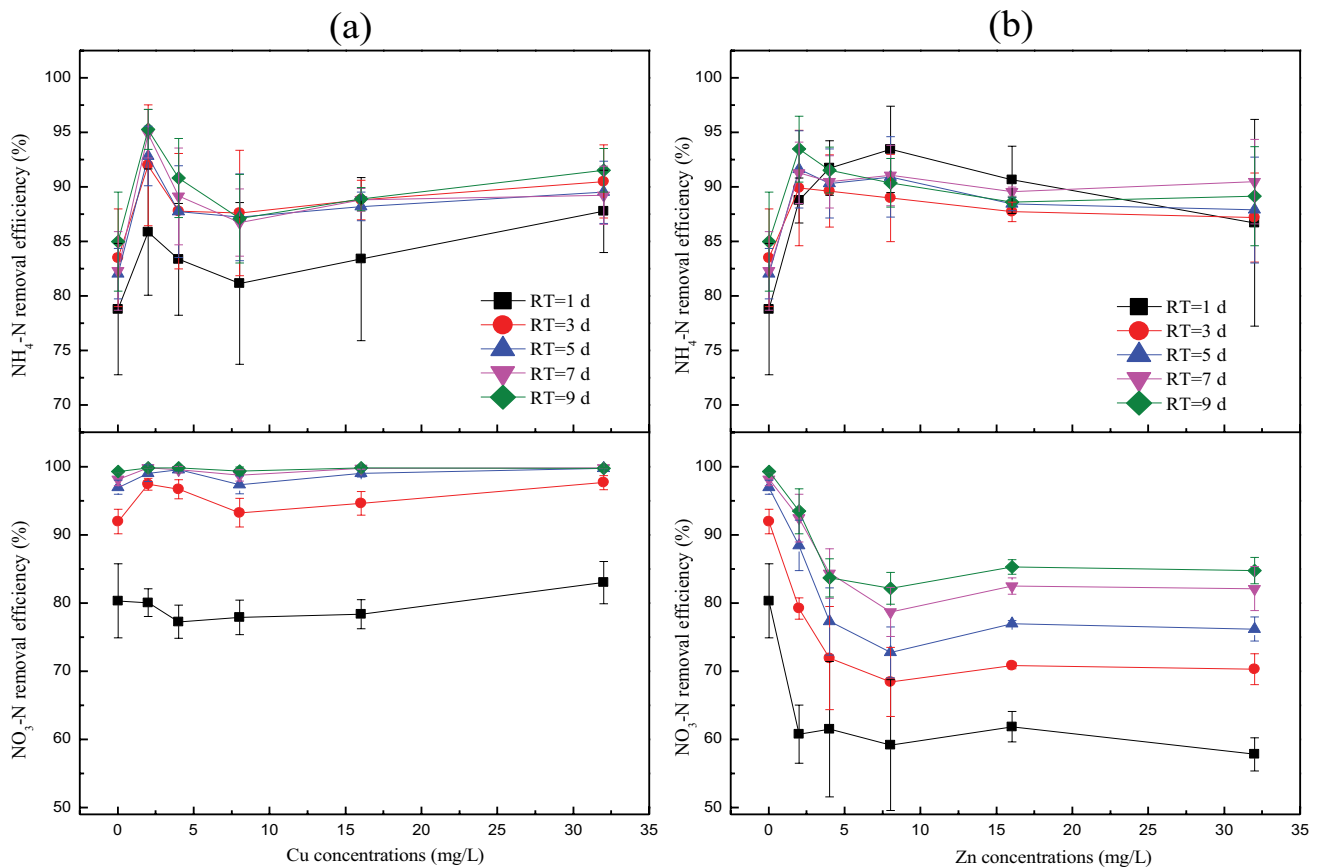Figs. 2 and 3 present variations in NH_4^+–N, NO_3^-–N and TN removal with the applied RT in all the treatments.

Fig. 2. NH_4^+–N and NO_3^-–N removal as a function of RT in HSSFs in the presence of different Cu^{2+} and Zn^{2+} concentrations.
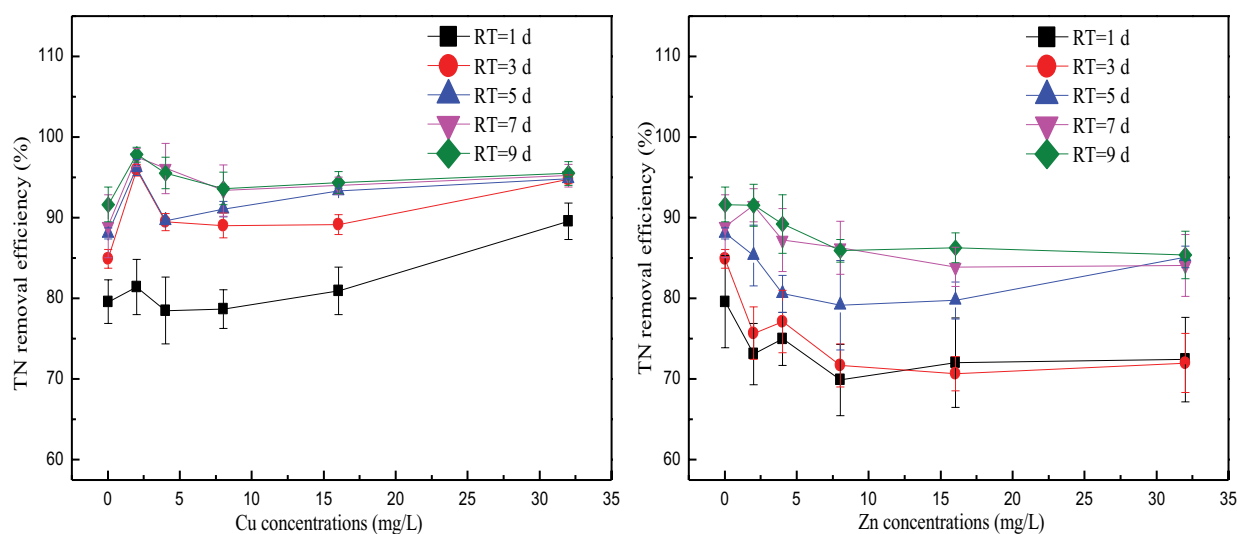
Fig. 3. TN removal with RT in HSSFs under different Cu^{2+} and Zn^{2+} additions.

Nitrogen was supplied principally as NH_4^+–N and NO_3^-–N in influent water. TN removal efficiencies were affected by both the NH_4^+–N and NO_3^-–N concentrations. An enhancement of the NH_4^+–N removal efficiencies was found in constructed wetlands with Cu^{2+} supplementation. That was because Cu can precipitate or co-precipitate with sulfur, iron and manganese in constructed wetlands [23], which increased the specific surface area of adsorbing NH_4^+–N and could promote NH_4^+–N removal. In addition, copper is an important constituent of some enzymes in the anaerobic ammonium oxidation (Anammox) bacteria, such as nitrite reductase [27], and is also an essential component for the microorganisms' survival and growth, and then short-term exposure (the exposure time of 9 d) in Cu^{2+} could improve Anammox process by increasing anaerobic ammonia-oxidizing bacteria bioactivity [24], which was beneficial to the NH_4^+–N removal in HSSF-CWs. However, high Cu^{2+} concentrations can inhibit nitrogen removal processes, and affect nitrification [27]. Uptake by *Phragmites australis* as well as adsorption and desorption of slag substrates also played a role on NH_4^+–N removal, which caused that the NH_4^+–N removal efficiencies increased, decreased and then sightly increased in HSSF-CWs with the Cu^{2+} concentration increasing. Hence, the variation of NH_4^+–N removal efficiencies in HSSF-CWs was affected by slag substrate adsorption and desorption, plant uptake, and microbial degradation.

The NH_4^+–N removal efficiencies were the lowest in the HSSF-CWs in the absence of Cu^{2+} (Fig. 2a). when the RT was 1 d, the NH_4^+–N removal efficiencies showed a certain fluctuation with the Cu^{2+} concentration increasing, and the largest value of the NH_4^+–N removal efficiency amounted to 87.78% at the Cu^{2+} concentration of 32 mg/L. When the RT was 3 d, the highest removal efficiencies of NH_4^+–N were shown at the Cu^{2+} concentration of 2 mg/L. At the Cu^{2+} concentration of 2 mg/L, the NH_4^+–N removal efficiency increased with the increasing RT, and was largest at the 7 d, which illustrated that the capacity of a horizontal subsurface flow constructed wetland system for the NH_4^+–N removal was higher under the Cu^{2+} concentration of 2 mg/L.

The greatest amount of NH_4^+–N removed was 95.3% in CWs with the Cu^{2+} concentration of 2 mg/L, suggesting that it was beneficial to removing NH_4^+–N. This was because the copper inhibition of nitrogen removal processes, especially Anammox process, played a vital role in NH_4–N removal at the level of more than 4 mg/L [27]. More precipitation with Cu^{2+} was produced by adsorption and co-precipitation with Fe and Mn oxides and stacked under high Cu^{2+} concentrations in constructed wetlands [28,29], and Cu was also retained by complexation by recalcitrant fractions of some organic matter in the constructed wetlands [30], and then the relative specific surface area of substrates in HSSF-CWs with high Cu^{2+} concentrations was larger than that in HSSF-CWs with low concentrations of Cu^{2+}, contributing to the higher NH_4^+–N removal efficiency in HSSF-CWs with the increasing Cu^{2+} concentrations.

For NO_3^-–N removal, in the presence of Cu^{2+}, when RT was more than 5 d, the removal efficiencies were greater. There was no significant difference on NO_3^-–N removal in all treatments from an RT of 5–9 d ($p > 0.05$), and the their values were close to 100%. At the RT of 1d, the NO_3^-–N removal efficiencies were more than 75% upon Cu^{2+} addition, and no significant difference of the removal was shown between HSSF-CWs without Cu^{2+} and HSSF-CWs with Cu^{2+} ($p > 0.05$), indicating that Cu^{2+} addition did not affect NO_3^-–N removal. Meanwhile, the removal efficiencies were significant greater on the RT of less than 3 d than that at the RT of 1 d with the increase in Cu^{2+} concentrations ($p < 0.05$) (Fig. 2a), showing an increase in NO_3^-–N removal with RT increasing, indicating that an increase in RT improved the NO_3^-–N removal.

In Fig. 2b, Zn^{2+} addition also enhanced the NH_4^+–N removal in all treatments from 2 to 16 mg/L. The positive removal of NH_4^+–N by the effect of Zn^{2+} was larger than that by Cu^{2+} in all treatments from 2 to 16 mg/L. This is because the faster response of anaerobic ammonia oxidizing bacteria (AAOB) to Cu^{2+} than to Zn^{2+} led to slighter inhibition of NH_4^+–N removal by Zn^{2+} than Cu^{2+} [24,27]. And low Zn^{2+} concentrations were favorable for ammonia-oxidizing bacteria

bioactivity, while the long-term effect also induced nitrite oxidizing bacteria bioactivity [31]. The effect of Zn^{2+} and Cu^{2+} on the NH_4^+–N removal was similar at the concentration of 32 mg/L, causing a decrease of NH_4^+–N removal efficiencies in the CWs with the Zn^{2+} concentration of 32 mg/L, indicating that the NH_4^+–N removal was limited by high concentrations of Zn^{2+} (32 mg/L). However, the effect of Zn^{2+} on the NH_4^+–N removal was stronger than that of Cu^{2+} in the all treatments at the RT of 1 d, and the greatest efficiency (93.45%) was found at a Zn^{2+} concentration of 8 mg/L at the RT of 1 d. This could be explained as Zn bound to the active site of ammonia monooxygenase, as a key enzyme of ammonium oxidation, and stimulated the growth and metabolic activities of aerobic ammonia oxidizing bacteria [32,33]. Under the presence of Zn^{2+}, the removal efficiency of NH_4–N in all the treatments increased and then declined with the Zn^{2+} concentration increasing above 8 mg/L, while no significant difference between all treatments was found ($p > 0.05$) (Fig. 2b). From 4 to 32 mg/L, the lowest NH_4^+–N removal efficiency was shown in HSSF-CWs with the Zn^{2+} concentrations (4–16 mg/L) increasing at the RT of 3 d, while the greatest removal efficiencies (93.47%) was found at an RT of 7 d at a Zn^{2+} concentration of 2 mg/L, indicating that the optimum concentration was 2 mg/L at the RT of 7 d, and also showing that the NH_4^+–N removal was affected by both of the Zn^{2+} concentration and the RT.

Zn^{2+} clearly inhibited the NO_3^-–N removal (Fig. 2b). When Zn^{2+} concentrations exceeded 2 mg/L, the removal efficiencies of NO_3^-–N in HSSF-CWs were below 86%, while the NO_3^-–N removal efficiencies in HSSF-CWs without of Zn^{2+} were the greatest. The removal efficiencies of NO_3^-–N in all treatments firstly decreased, and then remained stable with Zn^{2+} concentrations increasing. The removal efficiencies of NO_3^-–N in all treatments with Zn^{2+} increased with increasing RT, indicating that prolonging the RT was beneficial to the NO_3^-–N removal in the presence of Zn^{2+}. Until 7 d, the NO_3^-–N removal efficiencies increased with RT increasing, while their values were close between 7 and 9 d (Fig. 1b). Zn may be retained in wetlands by complexation by recalcitrant fractions of organic matter [30], which decreased the amount of carbon source for denitrification, thus limiting NO_3^-–N removal. With the recovery of dissolved oxygen by plant internal oxygen transport in HSSF-CWs, denitrification would also be inhibited, and NO_3^-–N would accumulate by the nitrification process [34]. Consequently, the removal efficiencies of NO_3^-–N started to decrease and then kept the stable tendency with Zn^{2+} concentrations increasing.

As shown in Fig. 3, on the whole, the efficiencies of the TN removal in CWs with Cu^{2+} concentrations were greater that in HSSF-CWs without Cu, illustrating the enhancement of nitrogen removal in the presence of Cu^{2+}. TN removal efficiencies increased with RT increasing in all treatments, and the lowest efficiency in HSSF-CWs with the Cu^{2+} concentrations increasing was found at RT of 1 d, indicating that an increase in RT was useful for nitrogen removal. TN removal efficiencies in HSSF-CWs with a Cu^{2+} concentration of 4 mg/L were lower than other treatments from 1 to 5 d, which was similar to the NH_4^+–N removal, showing that nitrogen removal was inhibited at a concentration of 4 mg/L during that period. This was because partial nitritation and Anammox was inhibited significantly by Cu^{2+} addition at the

moment, which affected nitrogen removal [27,35]. When Cu^{2+} concentrations were above 4 mg/L, slag substrate adsorption and denitrification played a role in the nitrogen removal. However, at the Cu^{2+} concentration of 2 mg/L, TN removal efficiencies in HSSF-CWs were the greatest from 3 to 9 d, indicating that the lower Cu^{2+} concentrations (≤ 2 mg/L) were useful for nitrogen removal in HSSF-CWs under short-term exposure to Cu^{2+}, and the result was consistent with Zhang et al. [24]; the impact of the Cu^{2+} concentrations (≥ 4 mg/L) on nitrogen removal was very small due to the precipitation or co-precipitation of Cu^{2+}.

The TN removal efficiencies were close between the RT of 1 and 3 d, and they were the lowest in all treatments with increasing Zn^{2+} concentrations (Fig. 3). The values of the TN removal efficiencies were the greatest and close at the RT of 7 and 9 d (approximately 84%~91.7%). A decrease of NO_3^-–N removal efficiencies was greater than a increase of NH_4^+–N efficiencies, which led to the decrease of the TN removal efficiencies in HSSF-CWs with Zn^{2+}. Compared with HSSF-CWs in the presence of Cu^{2+}, the removal capacity of TN in the presence of Zn^{2+} was lower ($p < 0.05$), mainly because Zn inhibited the removal of NO_3^-–N in HSSF-CWs.

3.2. Phosphorus and COD removal at various influent Cu^{2+} and Zn^{2+} concentrations

The removal efficiencies of phosphorus showed certain fluctuation with Cu^{2+} and Zn^{2+} concentrations increasing under the same RT (Fig. 4). Cu^{2+} did not significantly affect the TP removal. However, the TP removal efficiencies showed a slight increase in fluctuation with Cu^{2+} concentrations increasing during 7 d (Fig. 4). This may be because Cu^{2+} formed very insoluble compounds with sulfur in wetland systems [23], and more precipitation and soil salt was produced with Cu^{2+} increasing, which could increase the relative specific surface area of wetland substrates in HSSF-CWs and increased the adsorption sites for phosphorus to promote the TP removal. However, they remained a steady trend with Cu^{2+} concentrations increasing at an RT of 9 d, indicating that TP removal was not affected by the amount of Cu added to the influent at the RT of 9 d. The presence of Zn^{2+} had almost no effect on phosphorus removal. The values of TP removal efficiencies were 96.27%~99.18% in HSSF-CWs with Zn^{2+}, and their values were 97.55%~98.68% in HSSF-CWs without Zn^{2+}. Phosphorus might be adsorbed and desorbed from substrates in wetland systems during the RT of 9 d, leading to fluctuating changes in TP removal efficiencies with increasing Zn^{2+} concentrations. With the RT increasing, the removal efficiencies of TP were not significant different in all treatments ($p > 0.05$), except for the RT of 5 d. The adsorption capacity of substrates might be stronger than their desorption capacity in HSSF-CWs under Zn^{2+}, resulting in the phenomenon that the phosphorus removal in wetlands at the RT of 5 d was greater than that of other treatments with Zn^{2+}.

COD removal efficiencies showed certain fluctuation with Cu^{2+} concentrations increasing, and the COD removal showed a slight decrease in HSSF-CWs with Cu^{2+} concentrations increasing as a whole, except for the Cu^{2+} concentration of 2 mg/L (Fig. 4). At the Cu^{2+} concentration of 2 mg/L, the COD removal efficiencies were greater than that of other treatments. COD removal efficiencies increased in
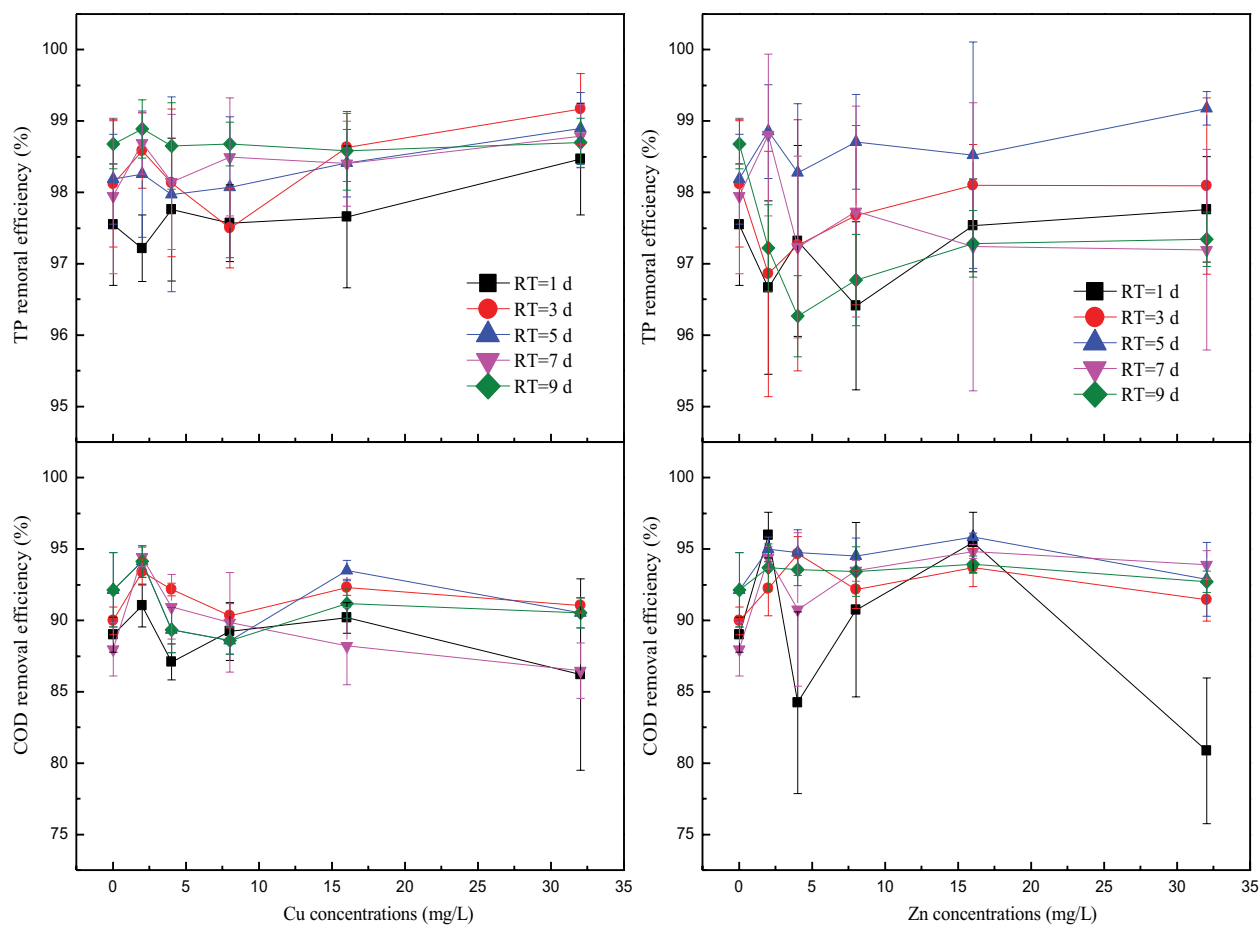
Fig. 4. TP and COD removal with RT in HSSFs under different Cu^{2+} and Zn^{2+} additions.

a fluctuating tendency under each Cu^{2+} concentration, and then they kept a stable trend from the RT of 3 d onwards, except for the RT of 7 d. At the RT of 7 d, the COD removal efficiencies decreased in HSSF-CWs with Cu^{2+} concentrations increasing. This was because, being a redox-active metal, Cu can catalyze reactive oxygen species (ROS) via Fenton-type reactions which may occur due to the release of ferrous ions from slag substrates containing 1.53%–2.06% ferric oxide (weight), leading to the damage of proteins, nucleic acids, lipids, and other biomolecules, thus affecting the activity of heterotrophic microorganisms [36], and then decreasing the COD removal efficiency.

In contrast, Zn^{2+} addition promoted COD removal in the HSSF-CWs, and COD removal efficiencies in almost all of HSSF-CWs with Zn^{2+} were greater than the control group at each RT (Fig. 4). This could be explained as heterotrophic microbial activity was enhanced in the presence of Zn^{2+}. This result is similar to that of Daverey et al. [22], and they found that the presence of Zn stimulated the microbial activities and COD removal performance. At the RT of 1 d, the values of COD removal efficiencies had great fluctuations with increasing Zn^{2+} concentrations, which indicated that the added amount of Zn^{2+} induced a great difference in the COD removal at the RT of 1 d. The values of COD removal were the greatest at the Zn^{2+} concentration of 2 mg/L, illustrating that the optimal Zn^{2+} concentration was 2 mg/L. The greatest

amount of COD removed in HSSF-CWs was 96.0% at the Zn^{2+} concentration of 2 mg/L (Fig. 4). The greatest values of COD removal were found at the Zn^{2+} concentration of 16 mg/L at an RT of 5 d (Fig. 4). However, RT had little influence on COD removal.

3.3. Change of Cu^{2+} and Zn^{2+} concentrations in HSSF-CWs

As shown in Fig. 5, Cu^{2+} concentrations decreased rapidly in HSSF-CWs with increasing RT, and the Cu^{2+} removal efficiency maintained a stable trend at the RT of 2 d. Cu^{2+} was detected in HSSF-CWs without Cu^{2+} addition. However, the Cu^{2+} concentrations were below 0.07 mg/L, indicating that some Cu^{2+} was released from wetland systems. Cu^{2+} concentrations in the influent water affected the Cu^{2+} concentrations in the effluent water. High influent Cu^{2+} concentrations led to higher Cu^{2+} concentrations in the effluent water. The Cu^{2+} concentration in the effluent water was below 0.076 mg/L at the concentrations of 16 and 32 mg/L at the RT of 7 d, and they decreased with RT increasing. Cu^{2+} concentrations in the effluent water showed a decrease in fluctuation in HSSF-CWs at influent Cu^{2+} concentrations ≤8 mg/L. Referring to the main removal mechanisms of Cu^{2+}, iron (Fe) oxides (1.53%–2.06%) adsorbed and coprecipitated with Cu^{2+} in HSSF-CWs, and Cu^{2+} also reacted with sulfur ions to produce cupric and cuprous sulfides that are insoluble compounds
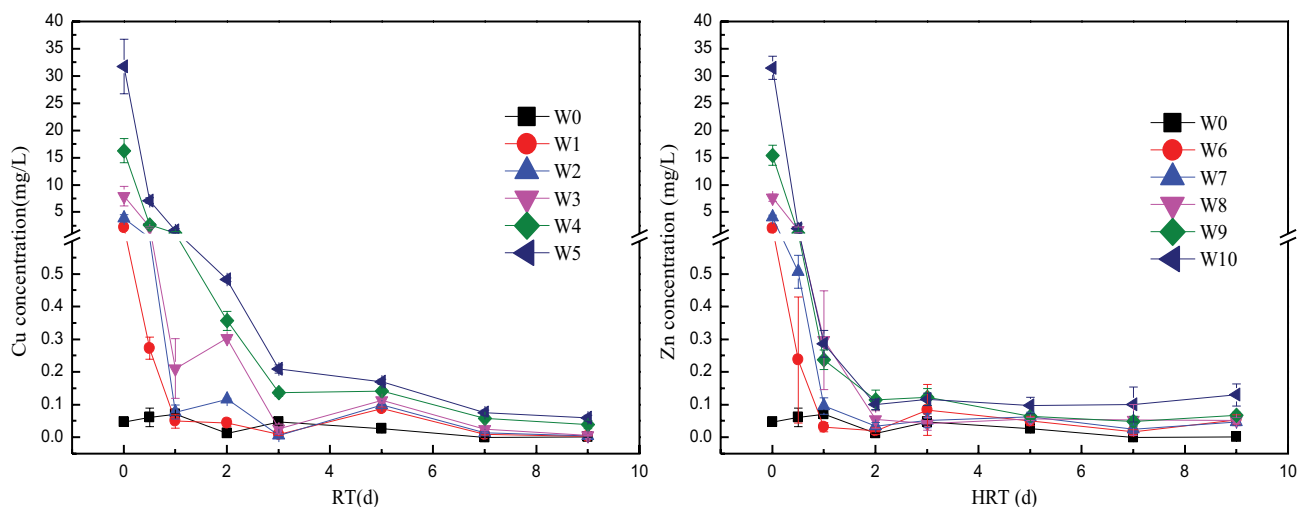
Fig. 5. The change of Cu^{2+} and Zn^{2+} concentrations with RT in HSSFs under different Cu^{2+} and Zn^{2+} additions.

in HSSF-CWs [23]. In addition, Cu^{2+} in HSSF-CWs may be retained by complexation by recalcitrant fractions of some organic matter types [30].

The values of Zn^{2+} concentrations in HSSF-CWs without Zn^{2+} addition were 0.027~0.060 mg/L at an RT of 9 d, which indicated that Zn^{2+} was released from the wetland systems when a presence of Zn^{2+} was absent from the feed (Fig. 5). Referring to the main removal mechanisms of Zn^{2+}, Zn was an essential element for the normal growth and metabolism of plants in HSSF-CWs [37], and then Zn^{2+} could be absorbed by *Phragmites australis* in HSSF-CWs, and be retained on iron plaques at the root surface of plants [38], Moreover, it could co-precipitate with Fe and Mn oxides [23]. For these reasons, a decrease tendency of Zn^{2+} concentrations occurred during 0–1 d, and then the Zn^{2+} concentrations remained a stable trend, indicating that the physical and biochemical reactions of Zn^{2+} are faster than those of Cu^{2+}. Zn^{2+} concentrations in the influent water also affected the Zn^{2+} concentrations in the effluent water. Zn^{2+} concentrations in the effluent water were greater than the Cu^{2+} concentrations in the effluent water under high influent concentrations at an RT of 7–9 d. That was because the solubility of Zn^{2+} in water was better than that of Cu^{2+} in HSSF-CWs [39].

3.4. ORP and pH changes in HSSF-CWs under various influent Cu^{2+} and Zn^{2+} concentrations

The value of ORP which represented the change of the oxidation–reduction condition in HSSF-CWs without Cu^{2+} addition were 524.6–576.2 mV (Fig. 6), and the oxidation–reduction condition affect the bioavailability of nutrients and trace metals in constructed wetlands by affecting chemical and microbial processes [40]. There was no significant difference in ORP in between HSSF-CWs without and with Cu^{2+} addition before 3 d. However, the ORP in HSSF-CWs fed with the influent containing Cu^{2+} was greater than that in HSSF-CWs without Cu^{2+} from 5 d ($p < 0.05$), and the greatest ORP values occurred during 5 d and 9 d, which showed that the capacity of the oxygen release from the plants' roots was enhanced by the presence of Cu^{2+}, because Cu is

an essential micronutrient. On the whole, ORP increased in a fluctuating tendency in the HSSF-CWs with the presence of Cu^{2+}. The ORP in HSSF-CWs with the presence of Zn^{2+} was larger than that in HSSF-CWs without Zn^{2+} (W0, $p < 0.05$). When Zn^{2+} concentrations exceeded 2 mg/L, ORP values were more than 600 mV at an RT of 2 d. However, there were no significant difference in the ORP between HSSF-CWs with Zn^{2+} ($p > 0.05$). At an RT of 5 d, there were the greatest values of ORP in HSSF-CWs with Zn^{2+}, and their values exceeded 620 mV, since Zn, as an essential trace element for plant growth, improved the metabolic capacity of plants. Nevertheless, Hauck (1984) noted that denitrification occurred in the presence of available organic matter only when the ORP was in the range +350 – +100 mV [41], and then the high ORP could inhibit the denitrification in HSSF-CWs.

In HSSF-CWs without Cu^{2+} or Zn^{2+}, the pH values of the effluent were greater than those in HSSF-CWs with Cu^{2+} or Zn^{2+}, which showed that the presence of Cu^{2+} or Zn^{2+} decreased the values of pH in wetlands systems, and then the pH values of treatment wetlands were typically around 7.0–7.8, which was suitable for the growth of nitrifying bacteria and denitrifying bacteria [42,43]. The pH value in HSSF-CWs with Cu^{2+} was lower than that in W1, and there was no significant difference between constructed wetlands with Cu^{2+} before 1 d (p > 0.05), indicating that Cu^{2+} has little influence on the pH of the HSSF-CWs effluent during 0–1 d. The pH values in HSSFs with Cu^{2+} were lower than those in HSSF-CWs without Cu^{2+} after 1d. However, the pH values were not significantly different between HSSF-CWs with Cu^{2+} ($p > 0.05$). The pH in HSSF-CWs with Zn^{2+} concentrations > 16 mg/L was greater than that in HSSF-CWs with Zn^{2+} concentrations < 16 mg/L, illustrating that higher influent Zn^{2+} concentrations could increase the pH value in constructed wetlands. Zn^{2+} and Cu^{2+} was affected by plant uptake, substrate adsorption, chemical reaction and microbial utilization in constructed wetlands, which might lead to the similar pH values between HSSF-CWs with Cu^{2+} or Zn^{2+}. However, as one of the most important factors for metal in environment, the pH has the strong effects on solubility and mobility of metals [44,45].
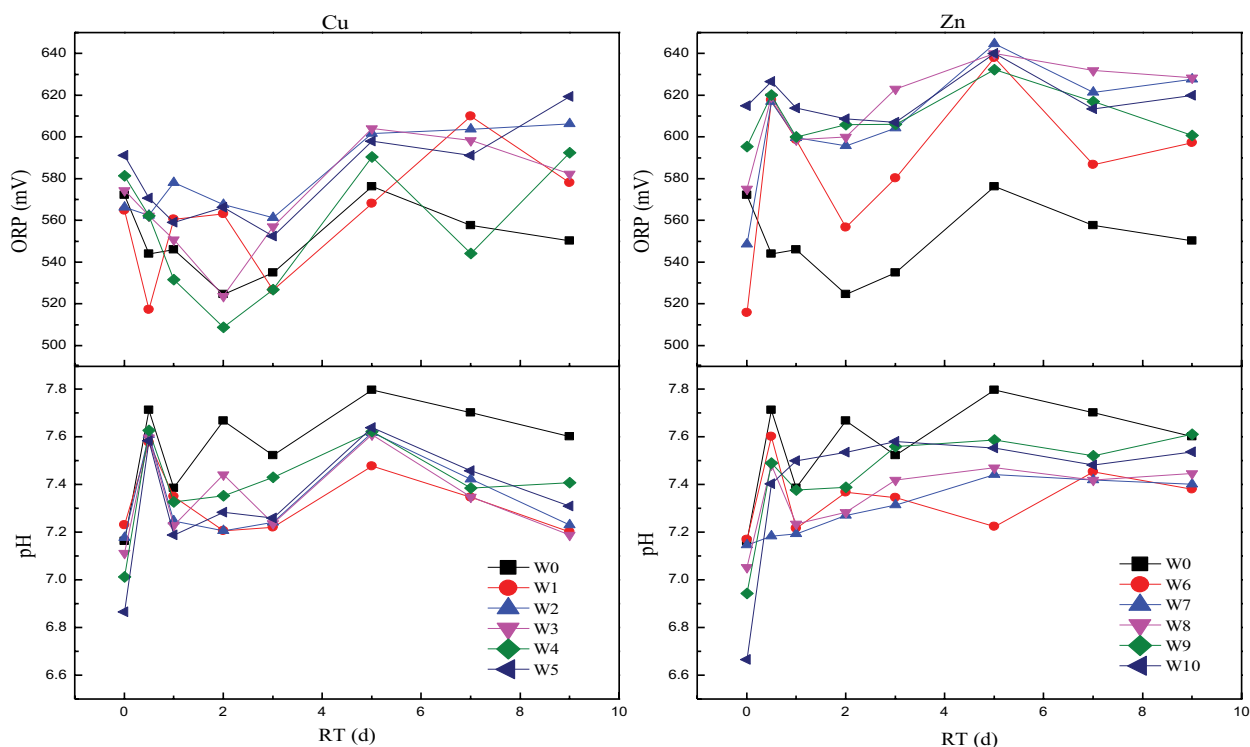
Fig. 6. The change of ORP and pH with RT in HSSFs under different Cu^{2+} additions.

4. Conclusions

In HSSF-CWs, the presence of Cu^{2+} could enhance nitrogen removal, especially TN and NH_4^+–N removal, and the optimal Cu^{2+} concentration was 2 mg/L. The greatest removal efficiencies of TN occurred at the RT of 7d in all HSSF-CWs with Cu^{2+}. However, Zn^{2+} inhibited the nitrogen removal, especially for TN and NO_3^-–N removal, and the removal efficiencies of their maximum value was at the RT of 9 d. The presence of Cu^{2+} and Zn^{2+} had no significant effects on TP and COD removal, while Zn^{2+} may partly increase the COD removal efficiencies. Zn^{2+} also significantly increased ORP of effluent water, while the presence of both Cu^{2+} and Zn^{2+} decreased pH values of the effluent.

Data availability

The data used and/or analyzed during the current study are available from the corresponding author, Xinhua Li, on reasonable request.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] J. Vymazal, Removal of nutrients in constructed wetlands for wastewater treatment through plant harvesting – biomass and load matter the most, Ecol. Eng., 155 (2020) 105962, doi: 10.1016/j.ecoleng.2020.105962.

[2] Q. Wang, C. Hernández-Crespo, M. Santoni, S. Van Hulle, D.P.L. Rousseau, Horizontal subsurface flow constructed wetlands as tertiary treatment: can they be an efficient barrier for microplastics pollution?, Sci. Total Environ., 721 (2020) 137785, doi: 10.1016/j.scitotenv.2020.137785.

[3] S. Singh, S. Chakraborty, Zinc removal from highly acidic and sulfate-rich wastewater in horizontal sub-surface constructed wetland, Water Sci. Technol., 84 (2021) 3403–3414.

[4] C. Liu, Y. Liu, C. Feng, P. Wang, L. Yu, D. Liu, S. Sun, F. Wang, Distribution characteristics and potential risks of heavy metals and antimicrobial resistant *Escherichia coli* in dairy farm wastewater in Tai'an, China, Chemosphere, 262 (2021) 127768, doi: 10.1016/j.chemosphere.2020.127768.

[5] Z. Zeng, P. Zheng, D. Kang, Y. Li, W. Li, D. Xu, W. Chen, C. Pan, The removal of copper and zinc from swine wastewater by anaerobic biological-chemical process: performance and mechanism, J. Hazard. Mater., 401 (2021) 123767, doi: 10.1016/j.jhazmat.2020.123767.

[6] National Animal Husbandry Service, How to Scientifically Understand the Influence of the Trace Elements Copper and Zinc in Feed, 2020. Available at: https://www.piyao.org.cn/2020-12/15/c_1210932198.htm

[7] Y. Zhao, J.Q. Su, X.L. An, F.Y. Huang, C. Rensing, K.K. Brandt, Y.G. Zhu, Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut, Sci. Total Environ., 621 (2018) 1224–1232.

[8] J. Li, Y. Xu, L. Wang, F. Li, Heavy metal occurrence and risk assessment in dairy feeds and manures from the typical

intensive dairy farms in China, Environ. Sci. Pollut. Res., 26 (2019) 6348–6358.

[9] Y. Xu, J. Li, Z. OU Yang, H. Zhang, Implications of feed mineral reduction and enhancement for China's feed standards, Resour. Conserv. Recycl., 168 (2021) 105342, doi: 10.1016/j.resconrec.2020.105342.

[10] X. Chen, H. Lin, Y. Dong, B. Li, T. Yin, C. Liu, Simultaneous high-efficiency removal of sulfamethoxazole and zinc(II) from livestock and poultry breeding wastewater by a novel dual-functional bacterium, *Bacillus* sp. SDB4, Environ. Sci. Pollut. Res. Int., 29 (2022) 6237–6250.

[11] J. Xu, G.H. Hao, Q. Jing, Y.Z. Zhai, Determination of Cu, Fe, Cr and Cd in wastewater from livestock and poultry farms, Agric. Environ. Dev., (2009) 74–75, 84.

[12] J. Zhang, Y. Wang, L. Ma, Y.L. Wen, S.Z. Chen, K.M. Yang, Z.J. Cai, Y.Q. Liao, Study on wastewater pollutant from planting and breeding recycled model, J. Southwest U Nationalities (Nat. Sci. Edit.), 37 (2011) 222–227.

[13] S.H. Ru, W.Q. Xu, S.Y. Sun, L.M. Hou, O.Y. Zhao, G.Y. Zhang, L. Wang, L. Liu, Distribution characteristics of nitrogen, phosphorus and heavy metals in intensive livestock and poultry wastewater in Hebei province, J. Hebei Agric. Sci., 25 (2021). 91–96.

[14] X. Liu, Y. Zhang, X. Li, C. Fu, T. Shi, P. Yan, Effects of influent nitrogen loads on nitrogen and COD removal in horizontal subsurface flow constructed wetlands during different growth periods of *Phragmites australis*, Sci. Total Environ., 635 (2018) 1360–1366.

[15] H. Xiang, X.Y. Yu, Toxic effect of copper pollution on water and hydrophyte, Hunan Agric. Sci., 11 (2009) 54–56 (in Chinese).

[16] X.F. Wang, The dangers of copper ions on the environment and research on countermeasure, Territory Nat. Resour. Stud., (2015) 55–57.

[17] B. Balen, M. Tkalec, S. Šikić, S. Tolić, P. Cvjetko, M. Pavlica, Ž. Vidaković-Cifrek Biochemical responses of *Lemna minor* experimentally exposed to cadmium and zinc, Ecotoxicology, 20 (2011) 815–826.

[18] M.R. Broadley, P.J. White, J.P. Hammond, I. Zelko, A. Lux, Zinc in plants, New Phytol., 173 (2007) 677–702.

[19] C.M. Palmer, M.L. Guerinot, Facing the challenges of Cu, Fe and Zn homeostasis in plants, Nat. Chem. Biol., 5 (2009) 333–340.

[20] J. Wang, The Study of the Rhizosphere Microorganisms of Poyang Lake Wetland Plant Under Zinc and Lead Stress, Master Thesis, Nanchang University, 2012 (in Chinese).

[21] M. Wu, Effect of ZNO-NPs on the Seedling Growth and Zn Absorption and Distribution of Different Plants, Master Thesis, Northwest A&F University, 2018 (in Chinese).

[22] A. Daverey, Y.C. Chen, S. Sung, J.G. Lin, Effect of zinc on anammox activity and performance of simultaneous partial nitrification, anammox and denitrification (SNAD) process, Bioresour. Technol., 165 (2014) 105–110.

[23] A. Galletti, P. Verlicchi, E. Ranieri, Removal and accumulation of Cu, Ni and Zn in horizontal subsurface flow constructed wetlands: contribution of vegetation and filling medium, Sci. Total Environ., 408 (2010) 5097–5105.

[24] X. Zhang, Z. Chen, Y. Zhou, Y. Ma, C. Ma, Y. Li, Y. Liang, J. Jia, Impacts of the heavy metals Cu(II), Zn(II) and Fe(II) on an Anammox system treating synthetic wastewater in low ammonia nitrogen and low temperature: Fe(II) makes a difference, Sci. Total Environ., 648 (2019) 798–804.

[25] C.L. Madeira, J.C. de Araújo, Inhibition of anammox activity by municipal and industrial wastewater pollutants: a review, Sci. Total Environ., 799 (2021) 149449, doi: 10.1016/j.scitotenv.2021.149449.

[26] Y. Zhang, X. Liu, C. Fu, X. Li, B. Yan, T. Shi, Effect of Fe²⁺ addition on chemical oxygen demand and nitrogen removal in horizontal subsurface flow constructed wetlands, Chemosphere, 220 (2019) 259–265.

[27] G.F. Yang, W.M. Ni, K. Wu, H. Wang, B.E. Yang, X.Y. Jia, R.C. Jin, The effect of Cu(II) stress on the activity, performance and recovery on the anaerobic ammonium-oxidizing (Anammox) process, Chem. Eng. J., 226 (2013) 39–45.

[28] J. Vymazal, P. Krása, Distribution of Mn, Al, Cu and Zn in a constructed wetland receiving municipal sewage, Water Sci. Technol., 48 (2003) 299–305.

[29] A. Sobolewsky, A review of processes responsible for metal removal in wetlands treating contaminated mine drainage, Int. J. Phytorem., 1 (1999) 19–51.

[30] G. Du Laing, G. Van Ryckegem, F.M.G. Tack, M.G. Verloo, Metal accumulation in intertidal litter through decomposing leaf blades, sheaths and stems of *Phragmites australis*, Chemosphere, 63 (2006) 1815–1823.

[31] X. Zhang, Y. Zhou, N. Zhang, K. Zheng, L. Wang, G. Han, H. Zhang, Short-term and long-term effects of Zn(II) on the microbial activity and sludge property of partial nitrification process, Bioresour. Technol., 228 (2017) 315–321.

[32] S. Gilch, O. Meyer, I. Schmidt, A soluble form of ammonia monooxygenase in *Nitrosomonas europaea*, Biol. Chem., 390 (2019) 863–873.

[33] S. Lee, K. Cho, J. Li, W. Kim, S. Hwang, Acclimation and activity of ammonia-oxidizing bacteria with respect to variations in zinc concentration, temperature, and microbial population, Bioresour. Technol., 102 (2011) 4196–4203.

[34] H. Zhu, B. Yan, Y. Xu, J. Guan, S. Liu, Removal of nitrogen and COD in horizontal subsurface flow constructed wetlands under different influent C/N ratios, Ecol. Eng., 63 (2014) 58–63.

[35] Q. Guo, Z.J. Shi, J.L. Xu, C.C. Yang, M. Huang, M.L. Shi, R.C. Jin, Inhibition of the partial nitritation by roxithromycin and Cu(II), Bioresour. Technol., 214 (2016) 253–258.

[36] L.H. Madkour, Function of reactive oxygen species (ROS) inside the living organisms and sources of oxidants, Pharm. Sci. Anal. Res. J., 2 (2019) 180023.

[37] G.R. Rout, P. Das, Effect of metal toxicity on plant growth and metabolism: I. Zinc, Sustainable Agric., (2009) 873–884.

[38] M.L. Otte, C.C. Kearns, M.O. Doyle, Accumulation of arsenic and zinc in the rhizosphere of wetland plants, Bull. Environ. Contam. Toxicol., 55 (1995) 154–161.

[39] M. McBride, S. Sauve, W. Hendershot, Solubility control of Cu, Zn, Cd and Pb in contaminated soils, Eur. J. Soil Sci., 48 (1997) 337–346.

[40] X. Xu, G.L. Mills, Do constructed wetlands remove metals or increase metal bioavailability?, J. Environ. Manage., 218 (2018) 245–255.

[41] R.D. Hauck, Atmospheric Nitrogen Chemistry, Nitrification, Denitrification, and Their Relationships, O. Hutzinger, Ed., The Handbook of Environmental Chemistry, Vol. 1. Part C, The Natural Environment and Biogeochemical Cycles, Springer-Verlag, Berlin, 1984, pp. 105–127.

[42] I. Burth, J.C.G. Ottow, Influence of pH on the Production of N₂O and N₂ by Different Denitrifying Bacteria and Fusarium Solani, Ecological Bulletins, No. 35, Environmental Biogeochemistry, Oikos Editorial Office, 1983, pp. 207–215.

[43] A. Princic, I. Mahne, F. Megušar, E.A. Pau, J.M. Tiedje, Effects of pH and oxygen and ammonium concentrations on the community structure of nitrifying bacteria from wastewater, Appl. Environ. Microbiol., 64 (1998) 3584–3590.

[44] H. Wang, X. Yuan, Y. Wu, H. Huang, G. Zeng, Y. Liu, X. Wang, N. Lin, Y. Qi, Adsorption characteristics and behaviors of graphene oxide for Zn(II) removal from aqueous solution, Appl. Surf. Sci., 279 (2013) 432–440.

[45] J. Liang, Z. Yang, L. Tang, G. Zeng, M. Yu, X. Li, H. Wu, Y. Qian, X. Li, Y. Luo, Changes in heavy metal mobility and availability from contaminated wetland soil remediated with combined biochar-compost, Chemosphere, 181(2017) 281–288.