Enhanced Nitrogen and Phosphorus Removal by Natural Pyrite based Constructed Wetland with Intermittent Aeration

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**Enhanced nitrogen and phosphorus removal by natural pyrite based
constructed wetland with intermittent aeration**

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Abstract: Four subsurface flow constructed wetlands (SFCWs) filled with different substrates including ceramsite, ceramsite+pyrite, ceramsite+ferrous sulfide, and ceramsite+pyrite+ferrous sulfide (labeled as SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4) were constructed, and the removal of nitrogen and phosphorus by these SFCWs coupled with intermittent aeration in the front section was discussed. The key findings from different substrate analyses, including nitrification and denitrification rate, enzyme activity, microbial community structure, and the X-ray diffraction, revealed the nitrogen and phosphorus removal mechanism. The results showed that the nitrogen and phosphorus removal efficiency for SFCW-S1 always remains the lowest, and the phosphorus removal efficiency for SFCW-S4 is recorded as the highest one. However, after controlling the dissolved oxygen by intermittent aeration in the front section of SFCWs, the nitrogen and phosphorus removal efficiencies of SFCWs-S2 and S4 becomes higher than those of SFCW-S1, and SFCW-S3. It was noticed that the pollutants were removed mainly in the front section of the SFCWs. Both precipitation and adsorption on the substrate were the main mechanisms for phosphorus removal. A minute difference of nitrification rate and ammonia monooxygenase activity was observed in the SFCWs aeration zone. The denitrification rates, nitrate reductase, nitrite reductase, and electron transport system activity for SFCW-S2 and SFCW-S4 were higher than those detected for SFCW-S1 and SFCW-S3 in the non-aerated zone. *Proteobacteria* was the largest phyla found in the SFCWs. Moreover, *Thiobacillus* occupied a large proportion found in SFCW-S2, and SFCW-S4, and it played a crucial role in pyrite-driven autotrophic denitrification.

Keywords: Constructed wetlands; Intermittent aeration; Autotrophic denitrification; Enzyme activity; Microbial community structure; Canonical correspondence analysis

Introduction

Urban inland rivers in China are mostly seasonal rivers, the water quality of these rivers is often affected by the sewage treatment plants effluent intrusion and rainstorm runoff mixing. As a result, these seasonal rivers gradually form black and smelly water bodies, called low pollution water, which is a great threat to ecological environment sustainability and human water safety concerns. In recent years, the purification of low pollution water polluted by the sewage treatment plants effluent intrusion and storm runoff mixing has attracted extensive attention for researchers.

Constructed wetlands (CWs) are widely utilized for advanced wastewater treatment purpose due to their relatively low cost, low energy demand feasibility, ease of operation, and beautification effect (Abou-Kandil et al. 2021, Baldovi et al. 2021, Rizzo et al. 2020). Heterotrophic denitrification is the main denitrification mechanism in traditionally CWs, and carbon source is considered the main limiting factor for the denitrification process (Pan et al. 2020, Si et al. 2018, Tan et al. 2020, Wang et al. 2021). Theoretically, the estimated C/N ratio for heterotrophic denitrification is 3.74 (Anjali & Sabumon 2015, Chiu & Chung 2003). In the case of application of the CWs for low pollution water purification, the C/N ratio for most low pollution water is lower than the value reported above, which makes it challenging to meet the carbon source requirements of heterotrophic denitrification process. Thus results in limited biological denitrification process as well as poor in term of denitrification efficiency. To boost the denitrification process, carbon sources can be added to improve denitrification efficiency. Generally, it is suggested that the higher carbon content availability in the influent is conducive to converting nitrate or nitrite into nitrogen, which produces less N_2O . However, excessive organic carbon availability in the influent will lead to the increase of unused carbon

sources in the denitrification process, in which become a cause of secondary pollution spreading, reactor blockage, and high effluent turbidity.

Moreover, in case of autotrophic denitrification, denitrifying bacteria reduce NO_3^- -N to N_2 by using H_2 , sulfide, sulfur, and reductive iron as electron donors, and NO_3^- as electron acceptor. The compounds including CO_2 , HCO_3^- , CO_3^{2-} are used as inorganic carbon sources. The sludge yield in the autotrophic denitrification process remains comparatively low, and no additional organic carbon source is needed to accomplish the task, which can effectively remove NO_3^- -N from low C/N sewage. Previous studies have shown that elemental sulfur's autotrophic denitrification process will lead to a drop in solution pH and produce a large amount of sulfate (Li et al. 2020, Sahinkaya et al. 2011, Wang et al. 2020). Therefore, pyrite is considered a good choice for autotrophic denitrification, low cost, stable pH, and less sulfate formation. The production of sulfate in the process of FeS autotrophic denitrification also remains much lower, when compared with elemental sulfur autotrophic denitrification.

In the present study, subsurface flow constructed wetlands (SFCWs) with different substrates ceramsite (SFCW-S1), ceramsite+pyrite (SFCW-S2), ceramsite+ferrous sulfide (SFCW-S3), and ceramsite+pyrite+ferrous sulfide (SFCW-S4) were used for the purification of low pollution water, i.e., seasonal river water contaminated by the sewage treatment plant effluent and storm runoff. The main research objectives for this study were as follows: (1) comparing the purification efficiency of CWs filled with different substrates with and without intermittent aeration; (2) exploring the nitrogen and phosphorus removal effects of CWs filled with different substrates, as well as the nitrogen removal effects of ferrous sulfide and pyrite as electron donors; (3) analyzing the migration and transformation of nitrogen elements, microbial activity, and community structure in CWs, as well as the nitrogen and phosphorus removal mechanism.

98 **Materials and methods**

99 **Chemical Reagents**

100 Sulphuric acid, methanol, anhydrous sodium acetate, sodium nitrate, and potassium
101 dihydrogen phosphate (Analytical Reagent) were all purchased from Sinopharm
102 Chemical Reagents Co., Ltd, Shanghai, China. NADH (100%) is obtained from
103 Sigma-Aldrich, Germany. Formaldehyde solution, phenol, potassium chloride, sodium
104 chloride, sodium disulfite, sodium nitrite, ammonium sulfate, anhydrous sodium
105 dihydrogen phosphate, D-anhydrous glucose, disodium hydrogen phosphate, potassium
106 chloride, iodonitrotetrazolium chloride (INT, 98%), and N-allyl thiourea (Analytical
107 Reagent) were produced by Shanghai Macklin Biochemical Co., Ltd, China.

108 **Experimental apparatus and methods**

109 Total four SFCWs labeled as SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were
110 constructed having same dimensions (length×width×height=80 cm×30 cm×60 cm). The
111 experimental apparatus diagram has been presented in Fig.1. Moreover, the filling
112 structure of substrates has given in Table 1. Three *cannas* plants were planted at equal
113 intervals in total of four SFCWs. The aeration pipe was located in the first chamber of the
114 four SFCWs. The total aeration time was 20 h per day, 8 cycles per day. The experiment
115 was carried out from September 1, 2020 to January 16, 2021. A certain amount of
116 compounds CH_3COONa , NH_4Cl , KNO_3 , and KH_2PO_4 was dissolved in the tap water to
117 prepare the synthetic wastewater. The influent water quality of the four SFCWs has been
118 given in Table S1.

119 **Analysis methods**

120 The conventional water quality index was analyzed by adopting standard methods
121 (Table S2). Biofilm denitrification rate (DNR) of the CWs substrates is calculated by the

decrease in NO_3^- -N concentration over time and divided by the initial biomass (Eq.(1)) (Dong & Reddy 2012, Zhang et al. 2017):

$$\text{DNR} = A/(T \times B) \quad (1)$$

where A is the amount of removed nitrate during the reaction (mg), T is the reaction time (h), and B is the amount of biomass used during the reaction (g).

The biofilm nitrification rate of the CWs substrates has represented by ammonium oxidation rate (AOR). AOR was calculated by the decrease in NH_4^+ -N concentration over time and divided by the initial biomass (Eq. (2)) (Zhang et al. 2017):

$$\text{AOR} = C/(T \times B) \quad (2)$$

where C is the amount of NH_4^+ -N removed during the reaction (mg), T is the reaction time (h), and B is the amount of biomass used during the reaction (g).

The determination methods of ammonia monooxygenase (AMO), nitrite reductase (NIR), and nitrate reductase (NAR) activities has been described in the previous study (Yang et al. 2020).

The electron transport system activity (ETSA) of the bacteria was determined by reducing 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT, a kind of exogenous electron acceptor) to formazan (INF). ETSA was calculated according to the following formula (Eq. (3)) (Yang et al. 2020):

$$\text{ETSA}(\mu\text{gO}_2(\text{g protein} \cdot \text{min})) = \frac{\text{ABS}_{490}}{15.9} \frac{V_1}{V_0 t} \frac{32}{2} \frac{1}{m} \quad (3)$$

where ABS_{490} is the sample absorbance, 15.9 is the specific absorptivity of INT-formazan, V_0 and V_1 are the initial volume of bacteria and the total volume of methanol (mL), t is the incubation time (min), 32/2 is the constant for the transformation of μmol INT-formazan to $\mu\text{g O}_2$, and m is the protein concentration per milliliter of bacteria (mg protein/mL bacteria).

X-ray diffraction (XRD) characterization of substrates was performed by the X-ray diffractometer (X'Pert Pro MPD, PANalytical, Netherlands).

Microbial community structure was determined by Sangon Biotech (Shanghai) Co., Ltd., China. The selected amplification area was 16S RNA V3-V4. The main process of metagenome sequencing was: sample DNA extraction, library construction and sequencing. DNA was extracted by DNA kit, and then the target sequences were enriched by highly specific primers. Finally, the sequencing data were analyzed by bioinformatics.

Results and discussion

Effects of SFCWs substrates on the removal of pollutants

The overall performance of SFCWs series for the removal of COD_{Cr} was monitored. The removal of COD_{Cr} by SFCWs having different substrates has presented in Fig. S1. As-obtained results from one-way ANOVA showed that there was no significant difference found for COD_{Cr} removal efficiency for all SFCWs before and after aeration ($p>0.05$). This endorsed that the substrates and aeration have little effect on the COD_{Cr} removal by SFCWs series.

Furthermore, as depicted in Fig. 2a, the effluent concentration of total nitrogen (TN) fluctuated between 1.44 and 10.18 mg/L. During initial stage in SFCWs, the adsorption and ion exchange of substrates were responsible for the removal of TN and NH₄⁺-N (Buelna et al. 2008, Yuan et al. 2020), which supported that the initial removal efficiency of TN and NH₄⁺-N was relatively higher (Fig. 2b and Fig. 2d). As the absorption of NH₄⁺-N by the substrate tends to be saturated, the abundance of ammonia-oxidizing bacteria in the system is not enough to oxidize NH₄⁺-N, and the removal efficiency of NH₄⁺-N begins to decline. As both COD_{Cr} and microorganism of SFCWs compete for dissolved oxygen (DO), so DO level in the wetland got decrease, then the conversion rate of NH₄⁺-N was slowed down and the removal efficiency of TN was reduced (Li et al.

2017, Sun et al. 2004). For 30-65 days, the influent concentration of TN was ranged from 17.56-22.86 mg/L, the influent load increased from range 0.32-0.39 g/d to 0.49-0.60 g/d. Both SFCW -S2 and S4 exhibited a relatively higher TN removal efficiency (35.74%-71.79), while SFCW-S1 and SFCW-S3 have shown comparatively low TN removal efficiency. In addition, SFCW-S1 has reflected a consistently low TN removal efficiency even before and after aeration.

Before aeration, the DO level in the wetland was 0-0.7 mg/L (Fig. S2), and the $\text{NH}_4^+\text{-N}$ average removal efficiencies for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 71.58%, 77.58%, 58.74%, and 48.36%, respectively, which suggested the moderate nitrification capacity at low DO level (Anjali & Sabumon 2015, Bernat et al. 2011). After 30-65 days, aeration was done to reduce the accumulated $\text{NH}_4^+\text{-N}$ in the wetland. After aeration, DO levels in the wetland increased dramatically (Fig. S2), which is favorable for the growth and metabolism of aerobic organisms (Yuan et al. 2020). The $\text{NH}_4^+\text{-N}$ concentration in effluent of the four SFCWs decreased significantly after aeration, which met the Class III standard of the Environmental Quality Standard for Surface Water (EQSSW) (GB 3838-2002, China), and the removal efficiency of $\text{NH}_4^+\text{-N}$ was basically above 90%. In addition to aeration, the reason for the high $\text{NH}_4^+\text{-N}$ removal efficiency during the later stage might be the good growth of plants, which released a large amount of oxygen as electron acceptor to promote the oxidation of $\text{NH}_4^+\text{-N}$ (Yuan et al. 2020).

Furthermore, there was no production of $\text{NO}_3^-\text{-N}$ detected in SFCW-S4 and SFCW-S3 during the period 0-30 days before aeration as reflected in Fig. 2e, and the denitrification rate was the highest. While the concentration of $\text{NO}_3^-\text{-N}$ in S1 effluent was 3.30-5.28 mg/L, and the denitrification rate was relatively the lowest. After the aeration of the wetland, the increase in DO level led to conversion of $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$, and the

concentration of NO_3^- -N in the effluent of the four SFCWs got increased. The NO_3^- -N can be removed by denitrification, partial denitrification, and anammox in constructed wetland. Because of the low carbon-nitrogen ratio in the influent, the NO_3^- -N concentration in wetland SFCW-S1 was higher due to lack of denitrification carbon source, while NO_3^- -N concentration in wetland SFCW-S2, SFCW-S3, and SFCW-S4 was lower, indicating that pyrite and ferrous sulfide in SFCW-S2, SFCW-S3, and SFCW-S4 play an essential role in the removal of NO_3^- -N.

It was noticed that the effluent NO_2^- -N concentration in the four SFCWs increased first and then gradually decreased as demonstrated in Fig. 2f. By comparing the overall four SFCWs, it was found that the effluent NO_2^- -N concentration in SFCW-S3 and SFCW-S4 was the highest among others. The accumulation of NO_2^- -N can be attributed to incomplete nitrification and partial denitrification. It has been stated by many recent studies that incomplete nitrification in CWs can lead to the increase of NO_2^- -N concentration in the effluent (Lin et al. 2020).

In addition, the average concentration of total phosphorous (TP) in influent was 2 mg/L as depicted in Fig. 2g. For the period, 0-30 days, the TP concentration in SFCW-S1 and SFCW-S3 effluent was low at the beginning. However, TP concentration gradually increased with increase in operating time and the removal efficiency of TP gradually decreased (Fig. 2h). The TP removal efficiency for the SFCW-S4 was the highest, and the effluent TP concentration (except for 5th day) could meet the Class III standard of the EQSSW (GB 3838-2002, China). The XRD peaks data presented in Fig. S3 reflected that the substrate of the four SFCWs primarily consisted of Si, Al, Fe, S, Ca, and Mg-bearing mineral phases, which indicates that the substrate had the potential for phosphate removal via precipitation and adsorption. The high TP removal efficiency of SFCWs was mainly due to the fact that the chemical precipitation formed by the reaction of metals with

phosphorus, and the adsorption by the metal oxides and hydroxides in the SFCWs substrate (Torrentó et al. 2010). The reactions were shown as follows (Eqs. (4)-(16)) (Ge et al. 2019, Omwene &Koby 2018).

Water quality changes along the water flow path

It was noticed that the effluent TN concentration gradually decreases along the water flow path of the SFCWs, and correspondingly, the TN removal efficiency gradually increases as shown in Fig. 3a and Fig. 3b. The highest TN removal efficiencies was noticed for SFCW-S2 and SFCW-S4, and the TN concentration for SFCW-S3 wetland was higher, when compared with other SFCWs. The TN removal potential for both SFCW-S2 and SFCW-S4 was much better than that for SFCW-S3 and control wetland SFCW-S1.

It was detected that $\text{NH}_4^+\text{-N}$ concentration in the sample was completely removed, when water reaches 15 cm away from the inlet point; the removal efficiencies found for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 84.47%, 96.51%, 96.34%, and 97.66%, respectively (Fig. 3c and Fig. 3d). Because this sampling place was very near to the wetland aeration port, the DO level was higher, which was supportive for the removal of $\text{NH}_4^+\text{-N}$ by nitrosation reaction. The average effluent $\text{NH}_4^+\text{-N}$ concentrations for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 1.18, 0.22, 0.27, and 0.26 mg/L, respectively. There was no significant difference for $\text{NH}_4^+\text{-N}$ removal efficiency among four SFCWs ($p>0.05$).

Furthermore, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations firstly increased and then decreased along the water flow path of the SFCWs as presented in Fig. 3e and Fig. 3f. In the first zone of the wetland (15 cm away from the water inlet), which is very closest to the aeration port, the higher DO content was found (Fig. S4), which was beneficial for the growth of nitrite and nitrifying bacteria, and existed $\text{NH}_4^+\text{-N}$ easily oxidized to $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$. However, with an increase of the water flow path, DO content gradually decreases (Fig. S4), which is conducive to accelerate the conversion of $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ to N_2 .

At the outlet point, 80 cm away from the water inlet, the concentration of $\text{NO}_3^-\text{-N}$ accumulated in SFCW-S1 and SFCW-S3 was higher than those determined for SFCW-S2 and SFCW-S4. Because the particle size of pyrite was much smaller than that of ferrous sulfide and ceramsite in the present study, substrate with small particle size could get good contact with microorganisms in wetlands and provide attached biological sites for the growth of microorganisms, thus accelerating the removal of $\text{NO}_3^-\text{-N}$ by autotrophic denitrifying bacteria (Bosch et al. 2011, Juncher Jørgensen et al. 2009, Torrentó et al. 2010). The reason for the low denitrification efficiency of SFCW-S3 might also be that

the particle size of ferrous sulfide was relatively large (Ye et al. 2011), the low solubility of ferrous sulfide decreased its utilization speed during autotrophic denitrifying process; furthermore, the coverage of biofilm on the ferrous sulfide surface hindered the contact between microorganisms and sulfur-containing substances (Li et al. 2016, Miot et al. 2011).

The average ratio of $\text{COD}_{\text{Cr}}/\text{NO}_3^-$ along the wetland was 1.82, 1.65, 2.00, and 2.41, respectively, which belongs to the low C/N ratio wastewater (Anjali & Sabumon 2015, Xu et al. 2018). No substrate containing sulfur or iron was introduced to SFCW-S1, the carbon sources were insufficient for heterotrophic denitrification, therefore, NO_3^- -N could not be effectively removed in SFCW-S1.

The TP concentration gradually decreases with the increasing flow path of the SFCWs, and the removal efficiency gradually increases (Fig. 3g and Fig. 3h). The TP removal efficiency for SFCW-S4 was above 90%, while the TP removal efficiency of SFCW-S1 was relatively low (<75%). The maximal removal efficiency for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 72.64%, 96.40%, 81.19%, and 97.88%, respectively. In the whole sampling site of the SFCWs (except 15 cm away from the water inlet), the removal efficiency of TP was in the following order SFCW-S4 > SFCW-S2 > SFCW-S3 > SFCW-S1. There were significant differences found in TP removal efficiency among the four SFCWs ($p < 0.05$), which indicated that the chemical reaction occurred between phosphorus and iron in the substrate, the adsorption of phosphorus by iron oxides and hydroxides in the substrate play an important role in the removal of aqueous phosphorus (Eqs.(4)-(16)).

Analysis for nitrification and denitrification rate

As shown in Fig. 4a, the nitrification rate of SFCW-S1 was the lowest for the aeration zone. The nitrification rate of SFCW-S2, SFCW-S3, and SFCW-S4 was

significantly different from that of SFCW-S1 ($p<0.01$), and the nitrification rates of SFCW-S2, SFCW-S3, and SFCW-S4 treatments were 1.37, 1.43, and 1.23 times higher than that of SFCW-S1, respectively. This indicated that the nitrification rates of SFCWs-S2, SFCWs-S3 and SFCWs-S4 containing inorganic electron donor (pyrite or ferrous sulfide) were significantly higher than that of SFCW-S1 without inorganic electron donor. The denitrification rate results for the non-aerated zone of SFCWs exhibited that the denitrification rate of SFCW-S2 and SFCW-S4 was significantly higher than that obtained for SFCW-S1 ($p<0.05$; Fig. 4b). However, there was no significant difference found between SFCW-S3 and SFCW-S1 ($p>0.05$). The denitrification rates for SFCW-S2 and SFCW-S4 were 2.31 and 2.22 times greater than calculated for SFCW-S1, respectively, suggesting that SFCW-S2 and SFCW-S4 had higher denitrification efficiency than SFCW-S1.

Analysis for microbial enzyme activity and electron transport system activity

Ammonia monooxygenase (AMO) is one of the key enzymes found in heterotrophic nitrification process, which assists in $\text{NH}_4^+\text{-N}$ conversion into $\text{NO}_3^-\text{-N}$ (Zhao et al. 2020). As shown in Fig. 4c, AMO activity in the aeration zone of SFCW-S2, SFCW-S3, and SFCW-S4 were 1.17, 1.21, and 1.26 times higher than the control group SFCW-S1, respectively. There was a significant difference between the AMO activity of SFCW-S2, SFCW-S3, SFCW-S4, and the control group SFCW-S1 AMO activity, respectively ($p<0.05$), which was similar to the nitrification rate result (Fig. 4a).

Denitrifying enzymes are responsible for the bio-reduction of $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$, NO and N_2O in the denitrification process (Wu et al. 2018, Zhao et al. 2020). Nitrate reductase (NAR) activity is critical for the denitrification process, reducing $\text{NO}_3^-\text{-N}$ to $\text{NO}_2^-\text{-N}$. Fig. 4d shows that the NAR activity for SFCW-S2 and SFCW-S4 was 1.52 and 2.08 times of that was calculated for SFCW-S1, and 1.41 and 1.93 times of that was

calculated for SFCW-S3, respectively, indicating that the decrease in NAR activity for SFCW-S1 and SFCW-S3 leads to the accumulation of NO_3^- -N (Wu et al. 2018). The NIR activity is also considered a key factor for denitrification. Fig. 4e shows that there was no significant difference found in NIR activity among SFCW-S2, SFCW-S3, SFCW-S4 and SFCW-S1 ($p>0.05$). However, the NIR activity for SFCW-S2 and SFCW-S4 was relatively higher than that obtained for SFCW-S1 and SFCW-S3. For SFCW-S2 and SFCW-S4, denitrification efficiency can be promoted by improving the activities of NAR and NIR, and then catalyzing the reduction of NO_3^- -N and NO_2^- -N, which is considered to be the reason for the increased TN removal efficiency and the decreased NO_3^- -N accumulation (Fig. 2b and Fig. 2e).

The efficiency for nitrification and denitrification process mainly depends on the efficient transport of electrons, that can be assessed using ETSA (Wan et al. 2016, Wu et al. 2018). AMO, NAR, and NIR obtained electrons from electron transport system, and the activities of these enzymes are directly related to nitrogen removal efficiency. The AMO, NAR, and NIR activity obtained for SFCW-S1 was comparatively low (Fig. 4c, Fig. 4d and Fig. 4e), which was an important reason for the low ETSA (Fig. 4f).

Analysis for microbial community structure

The structure and composition of microbial community in SFCWs at the phylum level has shown in Fig. 5a. The dominant bacterial community has divided into 13 phyla, *Proteobacteria* accounts for the highest relative abundance at each sampling point, ranging from 44.60% to 87.16%. *Proteobacteria* is usually the largest phyla found in constructed wetland systems (Guan et al. 2015, Ibekwe et al. 2016). *Proteobacteria* contain bacteria responsible for nitrification and denitrification activities and various metabolic bacteria, which play a vital role in the removal of organic matter, nitrogen, and phosphorus.

Cyanobacteria are often detected in high nutrient wastewater as an indicator bacterium (Adyasari et al. 2019), which was enriched in SFCW-S1 and SFCW-S4. *Cyanobacteria* with high relative abundance detected in SFCW-S1 indicated that the TN removal performance for SFCW-S1 was lower than that observed for the other SFCWs. Relative higher abundance of *Cyanobacteria* noticed for SFCW-S4 might be assigned to the accumulation of phosphorus in SFCW-S4 substrate and biofilm. Moreover, *Planctomycetes* contain bacteria related to Anammox (Bae et al. 2010). *Planctomycetes* not only has the function of Anammox, but also may play denitrification function (Du et al. 2020, Ishimoto et al. 2020, Kumar et al. 2020, Park et al. 2020, Vipindas et al. 2020). It was found that the proportion of *Bacteroidetes* has increased with the outbreak of cyanobacterial blooms and gradually became the dominant bacteria found in eutrophic water (Kolmonen et al. 2004, Rashidan & Bird 2001, Wu et al. 2007). No matter in an aerated zone or non-aerated zone, the relative abundance of *Bacteroidetes* in SFCW-S1 was much higher than that monitored for SFCW-S2, SFCW-S3, and SFCW-S4, respectively, indicating the poor performance of denitrification and phosphorus removal for SFCW-S1.

Fig. 5b shows the microbial community structure composition of the overall four SFCWs at the genus level. *Acinetobacter* commonly exists around the roots of CWs, and most of them are aerobic bacteria, which can metabolize by using ammonia, nitrogen and glucose. This is consistent with the fact that the proportion of *Acinetobacter* in the aeration zones of the overall four SFCWs was higher than that in the non-aerated zones. *Rhodobacter* is a kind of phototrophic bacteria related to Fe (II) oxidation and nitrogen removal (Chakraborty & Picardal 2013). Higher abundance of *Rhodobacter* detected in SFCWs containing iron compared with control SFCW-1 suggested that the addition of

pyrite and ferrous sulfide changed microbial community structure and then improved denitrification efficiency.

Thiobacillus has the potential to use the sulfur present in pyrite for autotrophic denitrification, and the proportions of *Thiobacillus* found in SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 0.26%, 13.00%, 0.20%, and 1.90%, respectively. This trend indicates that *Thiobacillus* played an important role for the autotrophic denitrification of SFCW-S2 and SFCW-S4.

Pseudomonas is a genus of denitrifying bacteria, which is the dominant group of nitrifying bacteria in CWs and plays an important role in heterotrophic denitrification (Nicomrat et al. 2006, Okada et al. 2005, Rios-Montes et al. 2017). *Pseudomonas* contributes to removing both COD_{Cr} and TN, and may also be the dominant genus for TP removal (Ge et al. 2019). The relative abundance of *Pseudomonas* for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 7.61%, 0.84%, 0.27%, and 1.99%, respectively, suggesting that *Pseudomonas* played an important role in wetland nitrogen and phosphorus removal for SFCW-S1.

Aerobic denitrifying microorganisms such as *Paracoccus*, *Pseudomonas*, *Rhizobium*, *Novosphingobium*, and *Sphingomonas* were detected in aerated zones of the overall four SFCWs. Bacterial denitrification is not strictly an anaerobic process, denitrification may also exist under aerobic conditions. Aerobic denitrifying microorganisms have potential for nitrification and denitrification process simultaneously. *Geobacters* are iron-reducing bacteria with organic matter as an electron donor (Ge et al. 2019, Leang et al. 2003), which contribute to the removal of COD_{Cr}. In both aerated and non-aerated zones, the proportion of *geobacters* in SFCW-S2, SFCW-S3, and SFCW-S4 was higher than that found in SFCW-S1, indicating that iron-reducing process was more dominant for SFCW-S2, SFCW-S3, and SFCW-S4 than for SFCW-S1.

It has been reported that *Paracoccus* is more suitable to live in the water environment having higher TN content (Pan et al. 2020, Zhang et al. 2020). The proportion of *Paracoccus* detected for SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 were 0.31%, 0.05%, 0.98%, and 0.16%, respectively (Fig. 5b). For SFCW-S3 and SFCW-S1, the proportion was relatively higher, while SFCW-S2 and SFCW-S4 were relatively low, indicating that the TN content for SFCW-S3 and SFCW-S1 were relatively higher, which was in-consistent with the results presented in Fig. 2a.

Canonical correspondence analysis

Canonical correspondence analysis (CCA) is a nonlinear multivariate direct gradient analysis method, which is commonly used to study the correlation between microbial community structure and water environmental factors (Ter Braak &Prentice 1988). Fig. 6 demonstrated the relationship among the microbial community at the phylum level and seven environmental factors. The correlation coefficient between the first and second axes of environmental factors was 0, which indicates that the analysis results are reliable. The principal components (AX1 and AX2) can explain 79.11% of the bacterial structure, with AX1 explaining up to 63.01% and AX2 up to 16.1% of the total variation. Among the environmental factors analyzed, water temperature T and $\text{NH}_4^+\text{-N}$ were positively correlated with the first ordering axis (AX1), while TP, COD_{Cr} , TN, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ were negatively correlated with the AX1.

Furthermore, temperature T, $\text{NH}_4^+\text{-N}$, and TP were positively correlated with the second sorting axis (AX2), while COD_{Cr} , TN, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ were negatively correlated the AX2. *Proteobacteria* was positively correlated with TP, COD_{Cr} , TN, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$, and negatively correlated with $\text{NH}_4^+\text{-N}$ and water temperature T. In addition, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, and *Actinobacteria* were positively correlated with water temperature T and $\text{NH}_4^+\text{-N}$ and negatively correlated with

COD_{Cr}, TN, NO₂⁻-N, and NO₃⁻-N. According to the correlation analysis of the AX1 and AX2, it can be noticed that NO₂⁻-N, TP, and water temperature T can significantly affect the microbial community at the phylum level.

Conclusion

The concentration of NH₄⁺-N in effluent drops rapidly after intermittent aeration in the front section of the SFCWs, and the removal efficiency of NH₄⁺-N gradually reaches over 90%. The removal efficiency for SFCW-S2 and SFCW-S4 exhibited better removal performance of TN and TP than wetland SFCW-S3 and wetland SFCW-S1. The average effluent TP concentration for wetland SFCW-S4 could meet the Class III standard of EQSSW (GB 3838-2002, China, and the phosphorus removal efficiency was the highest. Nitrification rate and AMO activity in aeration zone of overall four SFCWs were not significantly different. The denitrification rates, NAR, NIR, and ETSA of SFCW-S2 and SFCW-S4 were higher than those of SFCW-S1 and SFCW-S3 in the non-aerated zone. *Proteobacteria* accounts for the highest relative abundance found at each sampling point, ranging from 44.60% to 87.16%. Heterotrophic denitrification was the main process in wetland SFCW-S1, *Thiobacillus* plays an important role in pyrite-driven autotrophic denitrification process of wetland SFCW-S2 and SFCW-S4. NO₂⁻-N, TP, and water temperature T can significantly affect the microbial community at the phylum level.

Declarations:

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Fig. 1

Figure 1

Experimental apparatus diagram of subsurface flow constructed wetlands (A and B refer to the sampling points for microbial community determination; Sample A and B in SFCW-S4 are the mixture of A1+A2, and B2+B2, respectively)

Figure 2

Removal of TN (a, b), $\text{NH}_4^+\text{-N}$ (c, d), $\text{NO}_3\text{-N}$ (e), $\text{NO}_2\text{-N}$ (f), TP (g, h) by SFCWs filled with different substrates (HRT= 2d; IC refers to influent concentration; ID refers to influent loading; EC refers to effluent concentration; SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4 refer to SFCWs with different substrates: ceramsite (S1), ceramsite+pyrite (S2), ceramsite+ferrous sulfide (S3) and ceramsite+pyrite+ferrous

sulfide (S4); “Class III” and “Class V” refer to the Class III and Class V standard of the Environmental Quality Standard for Surface Water (GB 3838-2002, China))

Fig. 3

Figure 3

Variations in TN (a, b), NH₄⁺-N (c, d), NO₃⁻-N (e), NO₂⁻-N (f), TP (g, h) along the water flow path

Fig. 4

Figure 4

Nitrification rate in aeration zone (a), denitrification rate in non-aeration zone (b), AMO activity in aeration zone (c), NAR (d), NIR activities (e) and ETSA (f) in non-aeration zone of the SFCWs (* indicates significant difference between the experimental group (S2, S3 and S4) and the control group (S1), one-way ANOVA, * $p < 0.05$, ** $p < 0.01$)

Fig. 5

Figure 5

Microbial community composition of biofilm samples at the phylum level (a) and genus level (b) (S1-1, S2-1, S3-1, and S4-1 refer to sample in the aerated zone of the SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4, respectively; S1-2, S2-2, S3-2, and S4-2 refer to sample in the non-aerated zone of the SFCW-S1, SFCW-S2, SFCW-S3, and SFCW-S4, respectively)

Fig. 6

Figure 6

CCA sequencing of environmental factors and constructed wetlands bacteria at the phylum level

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