Nitrogen removal from summer to winter in a field pilot-scale multistage constructed wetland-pond system

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Keywords: CWs, MCWP, SFCW, total nitrogen, HSCWs

Posted Date: December 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-136829/v1>

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Abstract

Background

Single-stage constructed wetlands (CWs) has a single ecological service function and is greatly affected by temperature, which are general in removal of total nitrogen. Multistage hybrid CWs were proven to capable of enhancing removal of nitrogen. Therefore, this study aimed to explore the variation in nitrogen removal in the combined CWs-pond process from summer to winter and the contribution of plant harvesting and the functions of bacteria to nitrogen removal.

Methods

A pilot-scale multistage constructed wetland-pond system (MCWP) with the process of "the pre-ecological oxidation pond + the two-level horizontal subsurface flow constructed wetland (HSCW) + the surface flow constructed wetland (SFCW) and the submerged plant pond (SPP)" was used to treat actual polluted river water in the field. During the 124 days of operation, the nitrogen concentrations in the units influent and effluent of the system were measured every two days, and the plant height in HSCWs and SFCW was measured once per month. When the system operated stably to the 72nd day, the substrates in the CWs were sampled to analyze the bacterial community structure and composition.

Results

The concentration of total nitrogen (TN) in the MCWP gradually decreased from 3.46 mg/L to 2.04 mg/L, and the average removal efficiency of TN was approximately 40.74%. The SPP performed the best among all units, and the TN removal efficiency was as high as 16.08%. The TN removal efficiency was significantly positively affected by the daily highest temperature. A formula between the total TN removal efficiency and the highest temperature was obtained by nonlinear fitting. The TN removal load rate in the HSCWs was 2.7–3.7 times that of the SFCW. Furthermore, the TN transformed by *Iris pseudacorus* L. accounted for 54.53% in the SFCW.

Conclusion

We found that the significant positive correlation between the daily highest temperature and the total TN removal rate a field MCWP system. The SFCW, as an advanced treatment unit, increased the proportion of nitrogen removed by plant harvesting. The bacteria completed the nitrogen cycle in the SFCW, which had high-density planting, through a variety of nitrogen removal pathways.

Introduction

In recent years, with the development of the national social economy, the contradiction between the construction of material civilization and the development of the water environment has become increasingly prominent. The deterioration of the water environment and ecological environment has become more severe. Surface waters have become increasingly polluted by nitrate nitrogen (NO_3^- -N) and ammonia nitrogen (NH_4^+ -N) to varying degrees (Wilbers et al., 2014). According to monitoring data from recent decades, nitrogen concentrations in rivers throughout the world are increasing annually (Domangue and Mortazavi, 2018). Nitrogen pollution in aquatic environments is a major international concern due to its toxicity, tendency to cause eutrophication, and associated loss of oxygen and subsequent fish kills, among other effects (Haas et al., 2017; Paredes et al., 2019). Furthermore, nitrogen compounds in drinking water may transform into nitrites, which will lead to an increase in the risk of various diseases (including cancers) to humans (Sajedi-Hosseini et al., 2018). Therefore, the problem of nitrogen pollution in water, whether from the perspective of ecological balance or the impact on human health, merits more attention (Zhang et al., 2015; Smith et al., 2015).

As a near-natural ecological treatment technology, constructed wetlands (CWs) have been widely used in wastewater treatment due to their advantages of simplicity, ecological economy, high efficiency, low consumption and strong hydraulic load resistance. The NH_4^+ -N and total nitrogen (TN) removal efficiencies in river water contaminated by CWs reached 90% and 50%, respectively (Zheng et al., 2014; Huang et al., 2011). The near-natural ecological treatment technology represented by CWs is an effective way to solve the problem of nitrogen removal from polluted river water.

However, the CWs approach has some defects, including limited adsorption saturation, clogging, and a single ecological service function. These aspects limit the promotion and application of CWs to some extent. The NH_4^+ -N removal efficiency prevails over TN, which may not

meet the demand of purified water quality (Vymazal, 2013). At the same time, temperature is an important factor limiting the purification of water quality by CWs (Lu et al., 2006). There are a variety of nitrogen removal mechanisms in CWs, and microbial denitrification is widely considered the most important mechanism. Plant uptake, substrate adsorption, and ammonia volatilization are generally considered second-level contributions to nitrogen removal (Green, 1996; Vymazal, 2007). In view of the physiological and biochemical characteristics of denitrifying bacteria, 34–37 °C is a suitable temperature range to ensure that the denitrification reaction is carried out stably, which can ensure the growth activity of denitrifying bacteria. When the temperature was 15 °C, the denitrification reaction rate decreased significantly, and the reaction almost stopped at 5 °C (Lu et al., 2006). The biomass of heterotrophic bacteria showed obvious seasonal changes (Gao, 1998). The nitrogen removal efficiency in CWs in summer was significantly higher than that in winter (Reddy, 1985; Nie et al., 2006). Therefore, it is particularly necessary to ensure the removal effect in CWs and improve the overall temperature resistance in the process.

Multistage CWs can effectively decrease the disadvantages of single-stage CWs, such as the low removal efficiency of nitrogen and unstable efficiency caused by season (temperature). When the hydraulic load rate was 0.053 m³/(m²·d), the annual average removal efficiencies of NH₄⁺-N and TN for river wastewater by surface-vertical flow constructed wetlands were 54.0 ± 6.3% and 53.9 ± 6.0%, respectively, and these values were significantly better than those obtained for single-stage CWs (Zheng et al., 2014). There may be an inherent quantitative relationship between temperature and removal efficiency. At the same time, the removal effect in each unit in relation to purification of the actual water body is worth studying. Moreover, there is limited information regarding the testing of bacterial community structure changes in different units of multistage CWs.

Therefore, a field pilot-scale multistage constructed wetland-pond system (MCWP) was constructed to investigate the variation in nitrogen removal performance from summer to winter. The contribution of plant harvesting to nitrogen removal was also discussed. The following assumptions were made: (1) there was a quantitative relationship between nitrogen removal efficiency and temperature in the MCWP; (2) plants played an important role in the process of nitrogen removal. The purposes of this paper were to explore the relationship between nitrogen removal efficiency and temperature, quantify the correlation by formula fitting and make it possible to predict the TN removal efficiency according to the daily highest temperature from summer to winter in a field CW.

Materials And Methods

Construction of the MCWP

An MCWP system including a raw water tank (RWT), ecological pond (EP), oxidation pond (OP), first-level horizontal subsurface flow constructed wetland (FHSCW), second-level horizontal subsurface flow constructed wetland (SHSCW), surface flow constructed wetland (SFCW) and submerged plant pond (SPP) was designed. The process is shown in **Fig. 1**. Each unit was welded by 1-cm-thick UPVC boards, reinforced by stainless steel, and connected by 20-mm PVC pipes. The size of each unit, effective water depth, hydraulic retention time (HRT) and water inlet and outlet modes are shown in **Table 1**.

Table 1 The size of each unit, effective water depth, HRT and water inlet and outlet mode.

Unit	RWT	EP	OP	FHSCW	SHSCW	SFCW	SPP
Size/mm	Φ65×90	200×140×2000	630×300×1500	1000×260×1250	1000×260×1250	1000×800×500	500×300×1550
depth/m		1.8	1.53	1.35	1.2	0.45	1.5
HRT/d		0.25	1	0.57	0.57	1.73	1.15
Mode		Down in up out	Drop in up out	Up in down out	Up in down out	Drop in up out	Up in and up out

The substrate in the horizontal subsurface flow constructed wetland (HSCW) was designed in two layers, and the thickness of the lower layer was 1000 mm. The lower layer was designed in three sections. The middle part was 840 mm thick. A mixture of gravel and steel slag of φ10-20 mm with a mass ratio of 4:1 was used as the filler layer for microbial film hanging; both the inlet and the outlet sides were 80 mm thick, and φ20-40 mm gravel was laid to intercept residual particulate pollutants in the water body and reduce the clogging of the main part. The upper layer was 200 mm thick, and φ5-8 mm gravel was laid for the rooting and fixation of emergent plants. The substrate in the SFCW was designed in two layers. The lower layer was 100 mm thick, and φ10-20 mm gravel was used as the supporting layer; the upper layer was a 300-mm-thick soil layer, which was used for the rooting and fixation of emergent plants. A schematic diagram of the HSCW and SFCW is shown in **Fig. 2**.

Start-up and operation of the MCWP

The plants used in the experiment were transported from the local river. The plants were carefully dug out, washed clean and stored in tap water for 2 weeks. *Nymphaea L.* (1 plant) was planted in the EP, and *Myriophyllum verticillatum L.* was planted in the OP and SPP at densities of 26 plants/m² and 33 plants/m², respectively. In the HSCW and SFCW, *Iris pseudacorus L.* was planted at densities of 5 plants/m² and 35 plants/m², respectively. The experiment was operated from Jul. 29 to Nov. 30, 2019, totaling 124 days. The experimental water was taken from the Xiaoyi River, which is located in Baoding city, Hebei Province (coordinates: 115° 51' 36" E, 38° 46' 12" N). The experimental site was located in Rongcheng county, Baoding city, Hebei Province (coordinates: 115° 52' 12" E, 39° 3' N). Specifically, 300 L of river water was taken from the river into the RWT at 9 a.m. every day, and the flow rate was 160 mL/min and obtained using a peristaltic pump. The river water flowed from the EP to the SFCW based on the height difference. The water flowed from the SFCW into the storage water tank, and then it was pumped into the SPP. Then, the water was discharged to the municipal pipe. To increase the oxygen content in the OP and SFCW, the mode of drop water was adopted. Samples were taken once every two days, and pH was measured immediately. All samples were analyzed within 24 hours. The influent quality was as follows: permanganate index (COD_{Mn}) was 6.82±4.70 mg/L; total phosphorous (TP) was 0.1±0.041 mg/L; TN was 3.46±1.03 mg/L; NH₄⁺-N was 0.72±0.51 mg/L; suspended solids (SS) concentration was 17.62±12.44 mg/L; and pH was 8.31±0.21.

Methods of determination and analysis

TN was determined by the alkaline potassium persulfate digestion UV spectrophotometric method (HJ636-2012); NH₄⁺-N was determined by Nessler's reagent spectrophotometry (HJ535-2009). The calculation of nitrogen content in *Iris pseudacorus L.* was based on previous research (Luo et al., 2009).

In the HSCW and SFCW, three representative plants of high, medium and low were selected, and the plant height was measured once per month. At the end of the experiment, the plant growth was recorded, and the total amount of plant absorption during the experiment was as follows:

$$M = (M_1 + M_2) \cdot P \quad (1)$$

where M is the uptake of nitrogen per unit area of wetland, g/m²; M₁ and M₂ are the parts of plant height growth and new shoots at the end of the experiment, respectively, g/m²; and P is the nitrogen content in plants, mg/g. The calculation formula of wetland removal rate per unit area is as follows:

$$F = (C_1 - C_2) \cdot Q \cdot t / S \quad (2)$$

where F is the amount of nitrogen removal per unit area in the wetland, g/m²; C₁ and C₂ are the nitrogen concentrations in the units influent and effluent, respectively, mg/L; Q is the influent flow rate of wetlands, L/d; t is the operation time of wetlands, d; and S is the surface area of wetlands, m².

When the system operated stably to the 72nd day (Oct. 9), the substrates in the CWs were sampled to analyze the bacterial community structure and composition. The sampling points of steel slag and gravel mixed substrates were selected at the position of 500 mm from the bottom of the vertical line of the substrate layer in the FHSCW and SHSCW, and the soil sample was taken at the position 200 mm from the bottom of the vertical line of the substrate layer in the SFCW. The samples were stored in an ultralow temperature refrigerator at -80 °C. Specific methods were applied according to previous studies (Li, 2015; Tong et al., 2019). The V3-V4 region of the bacterial 16S rRNA gene was amplified according to Hai et al. (2014). The removal of low-quality sequences and the screening of high-quality sequences with a 97 % identity threshold followed the methods of previous studies (Liu et al., 2019; Liu et al., 2020; Wang et al., 2019).

Temperature data came from the web of weather (www.Tianqi.com). All statistical analyses were performed using Origin 2019 and SPSS version 26.0 software and were considered significant at the p< 0.05 level.

Results

TN removal

TN removal effect in the EP and OP

Because the experimental wastewater was collected from actual river water, the influent TN concentration fluctuated greatly (Zhang, 2016). EP and OP were pretreatment devices for this constructed wetland system and were mainly designed for removing SS from the river water (Guo et al., 2011). In fact, these devices did play important roles in removing SS (data not presented in this study). During the experiment, the influent TN concentration was 1.78–7.18 mg/L, the average concentration was 3.46 mg/L, and the water quality was inferior V water (GB3838-2002). The effluent concentration and the removal efficiency in EP were 3.18 ± 1.10 mg/L and $6.93 \pm 24.46\%$ ($P > 0.05$), respectively.

EP was the first treatment unit of the system, and the nitrogen in the influent tended to be removed by denitrification in the aerobic/anoxic environment formed by the different dissolved oxygen (DO) concentration distributions in the pond, which may be one of the reasons why EP had a better TN removal effect in the early stage of operation (the average removal efficiency was 26.36%), as shown in Fig. 3. In addition, the uptake of plants in EP played an important role in TN removal. In the later period of operation, as the air temperature decreased, the activity of microorganisms and the growth of plants were inhibited, and the TN removal rate in EP tended to generally decline. At the same time, the TN concentration in the effluent was higher than that in the influent. This result may also be related to the secondary release of nitrogen from plant residues due to the disturbance of flow.

The TN concentrations in the influent and effluent of OP were 3.18 ± 1.1 mg/L and 2.99 ± 0.98 mg/L, respectively. The TN removal efficiency was $6.93 \pm 24.46\%$ ($P > 0.05$). As shown in Fig. 4, in the early period, the purification effect in OP was poor, and the effluent TN concentration increased, which may have been related to the release of nitrogen into water after the death and decay of *Myriophyllum verticillatum* L. in OP. Within one month after Aug. 10, the TN removal rate was maintained at a relatively high level, up to 56%, which was mainly dependent on the microbial action in OP. With OP running to the later stage, the TN removal rate showed a downward trend due to the influence of temperature change.

TN removal effect in the FHSCW and SHSCW

The TN removal efficiency in the FHSCW fluctuated little. The TN concentrations in the influent and effluent of the FHSCW were 2.99 ± 0.98 mg/L and 2.80 ± 1.00 mg/L, respectively. As shown in Fig. 5, the overall removal effect was good. The removal efficiency was $4.60 \pm 22.1\%$ ($P < 0.05$). Regardless of the negative value, the removal efficiency was $18.20 \pm 12.50\%$. This result is because the interior of the FHSCW, especially the non-rhizosphere zone, was often in an anoxic state; thus, the removal effect of TN caused by denitrification in the wetland was obvious. In the later period, the removal effect was relatively less affected by temperature changes, indicating that the FHSCW had a certain thermal insulation effect.

The TN concentrations in the influent and effluent of SHSCW were 2.80 ± 1.00 mg/L and 2.60 ± 0.91 mg/L, respectively. There was a significant difference in the influent and effluent concentrations of the SHSCW ($P < 0.05$). The removal efficiency was $2.40 \pm 33.45\%$. Regardless of the negative value, the removal efficiency was $20.27 \pm 15.10\%$. The pH value in the effluent of the SHSCW was higher than 9 in Aug., which may have inhibited the growth and reproduction of denitrifying bacteria and led to the low removal rate of wetlands in the early period (Fig. 6). In general, the optimal pH range for denitrification was 7–8, and the denitrification rate was the highest when the pH was approximately 7.5. When the pH was more than 9, the denitrification rate decreased significantly (Lu et al., 2006), which was consistent with the results of the experiment. Therefore, ammonia volatilization was an important way to remove nitrogen in Aug. From the beginning of Sep., the pH value of the effluent in the SHSCW dropped below 9. By the middle of Sep., the microorganisms in the SHSCW gradually adapted to the wetland environment and grew rapidly. In addition, after the effluent in the FHSCW entered the SHSCW, the DO concentration was further reduced. The anoxic/anaerobic environment in the wetland promoted the denitrification effect. The highest TN removal efficiency was 63.49% at the end of Sep. With the SHSCW running until Oct., the TN removal rate showed a downward trend due to the influence of air temperature change.

TN removal effect in the SFCW and SPP

As shown in Fig. 7, the change rule of the removal efficiency in the SFCW was similar to that in the SHSCW. The TN concentrations in the influent and effluent of the SFCW were 2.60 ± 0.91 mg/L and 2.42 ± 1.03 mg/L, respectively. The removal efficiency was $2.40 \pm 33.45\%$ ($P < 0.05$). Regardless of the negative value, the removal efficiency was $22.28 \pm 14.79\%$. In general, the TN removal rate in the SFCW was good in Aug. and Sep. (the average removal rate was 27.78%), but as the operation period entered Oct., the TN removal efficiency decreased continuously (the average removal efficiency was 15.44%) due to the influence of air temperature changes.

The TN concentrations in the influent and effluent of the SPP were 2.42 ± 1.03 mg/L and 2.04 ± 1.17 mg/L, respectively. The removal efficiency was $16.08 \pm 26.92\%$ ($P < 0.05$). Regardless of the negative value, the removal efficiency was $28.99 \pm 19.75\%$. As shown in Fig. 8, the TN removal in the SPP could be divided into three stages: the first stage was from the beginning of the experiment (Jul. 31) to Aug. 19, which was the stage in which the removal effect increased, with the highest removal efficiency of 59.37%. Since the plants in the pond grew

gradually and the microbial action increased gradually, the removal efficiency showed an upward trend. The second stage was from Aug. 19 to Oct. 5, which was the stable period in the SPP. The average removal rate of the pond was 34.49%, with a maximum of 76.69%, due to the high temperature. The microorganism activity was strong at this stage, and the denitrification effect was obvious. On the other hand, plant growth in the pond made a certain contribution to the uptake of nutrients. In the third stage, from Oct. 5 to the end of the experiment (Nov. 30), the microbial activity and plant growth in the pond were inhibited by the change in temperature; thus, the overall removal rate of the pond showed a downward trend.

TN removal effect in the MCWP

To investigate the overall removal rate in the MCWP for raw river water, Fig. 9 was drawn. The TN concentrations of the influent and effluent in the MCWP were 3.46 ± 1.03 mg/L and 2.04 ± 1.17 mg/L, respectively. The removal rate was $40.74 \pm 28.84\%$ ($P < 0.05$). The overall change in TN in the effluent could be divided into three stages. The initial stage was the increasing stage, which was mainly due to the release of nutrients from the plant residues in the pond and the increase in the pH value in the effluent of the HSCW, resulting in the low activity of microorganisms; thus, the total TN removal efficiency of the system was relatively low. However, as the microorganisms gradually adapted to the wetland environment, denitrification was enhanced. The middle stage was a stable period (Aug. 5-Oct. 5). In this period, the temperature was relatively high, the microorganisms grew vigorously, the average removal efficiency was 60.93%, and the total effluent concentration met the class V water standard. In the later period, the removal rate showed a declining trend from summer to winter. In early and mid-Nov., the removal rate slightly increased twice, which may have been related to the slight increase in temperature.

The TN concentration in the effluent of each unit showed a similar fluctuating trend as that in the influent. There was a significant correlation between the TN concentrations in the influent and effluent ($P < 0.01$). The TN concentration was gradually reduced in the system, and the nitrogen removal performance was mainly reflected in the back of the system. The later the unit was, the higher the TN removal efficiency was. This result was because more DO was consumed in the degradation of organic matter in the front of the system, and with the gradual deepening of the influent, the environment in the system gradually changed from an aerobic environment to an anoxic/anaerobic environment, which was more conducive to denitrification reactions. In addition to DO, denitrification was affected by factors such as organic matter concentration and temperature. In general, the optimal C/N (carbon as COD_{Mn}) for denitrification is 4–5 (Hanaki et al., 1992). The average accumulation rate of NO_3^- -N was $3.3 \text{ g}/(\text{m}^3 \cdot \text{d})$ in tide flow constructed wetlands (TFCWs) at 15°C , and the C/N ratio was 4 (Zhi and Ji., 2014). In this experiment, the inflow C/N was 1.9, which indicated that the reason for the decreased TN removal efficiency in Oct. was not only the temperature but also the organic concentration.

According to Vymazal's classification of hybrid CWs (Vymazal, 2013), the FHSCW-SHSCW-SFCW in this experimental device belongs to the hybrid systems that include the SFCW. Various types of CWs provide different redox conditions that are suitable for enhancing the removal of TN (Vymazal, 2011). It was necessary to compare removal efficiency in terms of removed loadings. Vymazal's evaluation of the treatment performance of 60 hybrid CWs indicated that the most efficient system for the removal of TN was the hybrid CW, which included an SFCW stage in the treatment line and was able to remove an average of $4.24\text{--}5.12 \text{ g TN m}^{-2}\text{d}^{-1}$. Linear fitting results revealed that the removed load of TN accounted for approximately 77% of the influent load. In the FHSCW-SHSCW-SFCW, the influent TN load and removed load of the hybrid CW were $2.65 \text{ g TN m}^{-2}\text{d}^{-1}$ and $1.95 \text{ g TN m}^{-2}\text{d}^{-1}$, respectively. The removed load accounted for 74% of the influent load, which was consistent with the results of Vymazal. The difference between single horizontal flow CWs and hybrid systems with the SFCW stage was statistically significant ($p < 0.05$). This result was because in the SFCW, radial oxygen loss of emergent plants and atmospheric reaeration provided aerobic conditions for nitrification, while root exudates and plant litter provided additional carbon source conditions for denitrification.

NH_4^+ -N removal

Figure 10 shows that the influent NH_4^+ -N was $0.27\text{--}3.18$ mg/L during the experiment, and the average concentration was 0.72 mg/L. After Aug. 5, the effluent in the MCWP was stabilized in Class III. The NH_4^+ -N removal effect in each unit of the system differed greatly. The NH_4^+ -N concentrations and removal rates in the EP, OP, PHSFCW, SHSFCW, SFCW and SPP were 0.58 ± 0.42 mg/L and $24.11 \pm 17.63\%$; 0.54 ± 0.39 mg/L and $7.88 \pm 14.84\%$; 0.56 ± 0.52 mg/L and $9.44 \pm 20.62\%$; 0.51 ± 0.44 mg/L and $9.65 \pm 21.26\%$; 0.56 ± 0.51 mg/L and $-3.43 \pm 14.39\%$; and 0.57 ± 0.50 mg/L and $0.18 \pm 21.24\%$, respectively. In general, the removal efficiency of NH_4^+ -N in the EP was the best, while that in the SFCW and SPP was the worst, even though the effluent TN concentration was higher than that of the influent, which may have been related to the release of pollutants from the dead plant residues into the water. The total removal efficiency of NH_4^+ -N in the system was $22.02 \pm 25.97\%$, and the removal effect was mainly in the front of the system. The later the unit was, the lower the NH_4^+ -N removal efficiency was. The EP was the main unit of NH_4^+ -N removal because the content of DO in the front of the system was higher, which

promoted the conversion of nitrogen in the form of $\text{NH}_4^+\text{-N}$ into $\text{NO}_3^-\text{-N}$ through nitrification, thereby reducing the concentration of $\text{NH}_4^+\text{-N}$. As more DO was consumed in the front of the system by Organic matter degradation, the system environment gradually changed from an aerobic environment to an anoxic/anaerobic environment, which was not conducive to nitrification. The removal efficiency decreased and the concentration increased when the effluent in the OP entered the FHSCW. The reason may have been that the DO concentration of the HSCW was lower than that of the pond and the SFCW. The removal rate of $\text{NH}_4^+\text{-N}$ in the SHSCW was improved, probably because of ammonia volatilization and Anammox. In general, the SFCW had a better reoxygenation ability than the HSCW, which could have provided a good living environment for nitrifying bacteria (Fu et al., 2012). Therefore, it had a better effect on $\text{NH}_4^+\text{-N}$ removal, which was inconsistent with the results of this study. This may have been due to the poor hygienic conditions in the SFCW being prone to breeding mosquitoes and flies. In the form of water retention, the algae on the surface layer propagated in large quantities. The DO in the water decreased, resulting in an increase in the $\text{NH}_4^+\text{-N}$ concentration in the effluent of the SFCW.

Vymazal and Kropfelova (2008) calculated that the average removal load of ammonia nitrogen by 85 single vertical flow CWs was $2.07 \pm 2.03 \text{ g NH}_4^+\text{-N m}^{-2}\text{d}^{-1}$. Free-drain vertical flow CWs are aerobic due to intermittent feeding, which allows for oxygen diffusion into the filtration bed (Vymazal, 2007). The characteristics of vertical flow CWs greatly enhance the removal efficiency of $\text{NH}_4^+\text{-N}$. Vymazal's results (Vymazal, 2013) indicated that there was no significant difference between single vertical flow CWs and hybrid CWs with the SFCW ($2.34 \pm 3.31 \text{ g NH}_4^+\text{-N m}^{-2}\text{d}^{-1}$) ($P > 0.05$). However, in this study, the influent N load and removal load of the FHSCW-SHSCW-SFCW hybrid CW were $0.48 \text{ g NH}_4^+\text{-N m}^{-2}\text{d}^{-1}$ and $0.32 \text{ g NH}_4^+\text{-N m}^{-2}\text{d}^{-1}$, respectively. The removed load accounted for 66% of the influent load, slightly lower than that of Vymazal (78%). The removal load of $\text{NH}_4^+\text{-N}$ by the HSCW was significantly weaker than that of other wetland types. Therefore, the weak reoxygenation capacity of the SFCW in this study led to a lower removal load of the FHSCW-SHSCW-SFCW hybrid CW.

Plant height growth

Figure 11 shows that the plant height in the HSCW and SFCW increased significantly throughout the experimental process. The plant height in the planted emerged plant unit showed that $\text{SFCW} > \text{FHSCW} > \text{SHSCW}$. Compared with the HSCW, the average plant height in the SFCW was higher. On the one hand, this result may have been due to the high contents of organic matter, nitrogen and phosphorus in the upper soil of the SFCW, which was more conducive to the growth of plants; in contrast, all nutrients required by plants in the HSCW came from the polluted water body and lacked a certain amount of elements necessary for plant growth. On the other hand, due to the influence of steel slag in the HSCW, the pH in the effluent of the FHSCW and SHSCW was higher than 8.73 and 8.23 and 9.13 and 8.37 in Aug. and Sep., respectively, which may have been a main reason for the slow growth of plants in this period. Water hyacinths were inhibited from growing under highly alkaline conditions, with yellowing leaves, relatively small plants, and some plant deaths (Zhang et al., 2013). With increasing pH, the plant height, leaf width and biomass of water hyacinth decreased significantly (Wang et al., 1996). The plant height increased obviously in Oct., which indicated that *Iris pseudacorus* L. had a strong ability to adapt to low temperatures. In Nov., with a further decrease in temperature, plant metabolism and enzyme activity decreased, plant growth stopped, and some leaves began to turn yellow and wither.

Discussion

Effect of temperature on nitrogen removal

Because the influent of this experiment was the actual river water, the TN concentration in the influent fluctuated greatly. Fig. 12 shows that the TN removal efficiency was positively correlated with air temperature ($P < 0.01$). The change trend of removal efficiency was divided into three stages. The early period was a period of rapid growth (Jul. 29-Aug. 5). This stage was the reproduction and growth stage of the wetland microbial community, and the removal efficiency was 16.80-65.20 %. The daily average lowest temperature was 23 °C, and the daily average highest temperature was 30 °C. The middle period was the stable period (Aug. 5-Oct. 2), with a removal efficiency of 37.70-84.00 %, a daily average lowest temperature of 18 °C, and a daily average highest temperature of 30 °C. Due to the high temperature and vigorous growth of microorganisms in this period, the TN removal efficiency was maintained at a high level. The later period was the declining period (Oct. 2- Nov. 28), with a removal efficiency of 5-54.2 %, a daily average lowest temperature of 3 °C, and a daily average highest temperature of 14 °C. With decreasing temperature, the denitrification effect in the system fell accordingly due to the weakening of microbial activity. The total removal efficiency of TN and $\text{NH}_4^+\text{-N}$ was set as group A, and the lowest temperature and the highest temperature were set as group B. Then, canonical correlation analysis was performed. The correlation coefficient was 0.808 ($P < 0.01$), and $A = -0.930 \text{ TN total removal efficiency} + 0.126 \text{ NH}_4^+\text{-N total removal efficiency}$, and $B = -0.296 \text{ lowest temperature} - 0.724 \text{ highest temperature}$. The maximum temperature was the main factor affecting the total removal efficiency of TN. From the discussion in Section 3.1, it can be seen that the TN removal efficiency was higher and the impact of air temperature change was greater in the later units of the system. The nitrogen removal performance began to be affected by the temperature drop around Oct. 2 (the daily lowest temperature was 15 °C, and the

overall temperature showed a downward trend starting on this day). The TN removal efficiency in the FHSCW and subsequent units all showed an asynchronous decline, indicating that the denitrification bacteria, as the main nitrogen removal mechanism in these units, were significantly affected by temperature changes ($P < 0.05$).

Huang et al. (2000) obtained the rate constant (K_T) formula related to air temperature, T , and predicted the total Kjeldahl nitrogen concentration in wetland effluent using the formula. Because the NO_3^- -N concentration was very low, the formula could be used to predict the effluent TN concentration. However, the limitation of this formula was that it was suitable for predicting only the effluent TN concentration under different HRTs at a certain temperature. In this paper, under an HRT of 5.27 d, the influence of temperature change on TN removal efficiency was explored. The nonlinear fitting equation is shown in **Fig. 13**, and the relationship between the highest temperature and TN removal efficiency was obtained as follows:

$$y = 14.198 + 52.323e^{-\frac{(x-28.947)^2}{80.569}} \quad R^2=0.7192$$

where x is the highest daily temperature, $^{\circ}\text{C}$; y is the total TN removal rate in the MFCW, %.

The temperature change has a strong impact on denitrification. The TN removal efficiency in the experimental study of CWs for wastewater treatment increased with increasing temperature in the range of 22–32 $^{\circ}\text{C}$ (Yang et al., 1991). Low temperature affected the proliferation rate and activity of denitrifying bacteria. The denitrification rate decreased by 9 % per 1 $^{\circ}\text{C}$ decrease in temperature. The denitrification speed was very slow when the temperature was lower than 5 $^{\circ}\text{C}$ (Yang et al., 2009; Lu et al., 2006). At the same time, it further showed that plant uptake had little effect on TN removal because it Oct. was a rapid period of plant growth but the TN removal efficiency was reduced by temperature. The inhibition of denitrification would lead to the accumulation of NO_3^- -N in the TFCWs (Chang et al., 2014; Hu et al., 2014; Li et al., 2015). When the temperature increased from 4 $^{\circ}\text{C}$ to 12 $^{\circ}\text{C}$, the accumulation rate of NO_3^- -N decreased, indicating that the lower temperature restricted the denitrification reaction and promoted the accumulation of NO_3^- -N (Pang et al., 2015). In the experiment, the TN removal efficiency in the system decreased after Oct. (i.e., denitrification was inhibited). When the removal efficiency of NH_4^+ -N had an upward trend (i.e., the system still had good nitrification performance), the accumulation of NO_3^- -N was promoted.

There was a significant negative correlation between the NH_4^+ -N total removal efficiency and temperature ($P < 0.01$). The removal of NH_4^+ -N is mainly completed by two groups of chemoautotrophic bacterium through two processes. The first step is to strictly oxidize NH_4^+ -N to nitrite nitrogen (NO_2^- -N) by strict aerobe. Such bacteria have been identified as *N. europaea* in fresh water and soil, as well as *N. briensis*, *Nitrosovibrio* (*N. tenuis*) and *N. multiformis* in soil. The second step is to oxidize NO_2^- -N to NO_3^- -N by facultative aerobe. Only one *Nitrite-oxidizing bacteria* (NOB), i.e., *Nitrobacter winogradskyi*, was found in soil and fresh water, and *Nitrospira gracilis* was found in seawater. After Oct., the CWs still had good nitrification performance, which may have been due to the average water temperature being higher than the lowest temperature required for nitrification (5 $^{\circ}\text{C}$) (Lu et al., 2009). When the temperature was lower than 15 $^{\circ}\text{C}$, the metabolism of ammonifying bacteria was inhibited, and the growth activity was weak (Zhang et al., 2018), which reduced the ammonification efficiency of organic nitrogen, and nitrification was greater than ammonification; thus, the concentration of NH_4^+ -N was reduced. Pang et al. (2015) found that at 4 $^{\circ}\text{C}$, 8 $^{\circ}\text{C}$ and 12 $^{\circ}\text{C}$, the TFCW had a high and stable removal efficiency of NH_4^+ -N (93–96 %). In addition, the conversion rate of NH_4^+ -N showed little change in the TFCWs ($P < 0.05$), indicating that temperature had little effect on the conversion of NH_4^+ -N.

Contribution of plant harvesting to nitrogen removal

The growth of aquatic plants in the MCWP played an important role in nitrogen removal. On the one hand, nitrogen taken up by plants was used for their own growth and metabolic needs and converted into plant protein and organic nitrogen. Then, nitrogen was removed from the system through harvesting (Brix, 1997; Wu, 1995). Because the role of plant nitrogen uptake accounts for a small part of N removal in wetlands, the effect of nitrogen removal by plant harvesting was not obvious (Robert and Kadec, 2003; Kem and Idler, 1999). On the other hand, the soil volume touched by the developed roots of emergent plants in wetlands varied depending on the depth and extension patterns of roots (Zhao et al., 2006) and formed a well-diffused and large-scale physical environment in the rhizosphere to support the survival and reproduction of a large microbial community. In addition, root exudates increased the organic matter content in the substrate of the rhizosphere, which promoted the growth and reproduction of microorganisms. Plant roots can transport oxygen to the substrate, forming aerobic and anoxic microenvironments around the rhizosphere, which is conducive to nitrification and denitrification.

Because of the periodicity of plant growth, some people advocate regular harvesting of plants to prevent pollutant accumulation in the plant caused by the decomposition of plant residues, resulting in another release and causing secondary pollution (Jiang et al., 2013). Others believe that the organic matter released in the process of plant decomposition enters the water body. After that, it provides a certain carbon source for the anaerobic bacteria in the water body and sediment and promotes the denitrification effect (Fennessy et al., 2008; Chimney and Pietro, 2006). At the end of this experiment, the concentration of TN in the effluent was occasionally higher than that in the influent, which was caused by the release of $\text{NH}_4^+\text{-N}$ into the environment after the decomposition of plant roots, in addition to the denitrification limited by temperature. Qi and Liu. (2016) showed that the decomposition of *Phragmites australis* could increase the concentration of $\text{NH}_4^+\text{-N}$ in the effluent. The $\text{NH}_4^+\text{-N}$ concentration increased with increasing residue amount. Therefore, appropriate harvesting frequency, methods and timing are very important.

Table 2 Nitrogen mass balance.

Unit	FHSCW	SHSCW	SFCW
TN removal/(g/m ²)	17.08	23.57	6.29
Plant uptake/(g/m ²)	2.66	2.76	3.43
Percentage of plant uptake/(%)	15.57	11.71	54.53

During the experiment, the influent nitrogen load rate in the FHSCW was 321.95 g/m².

The nitrogen mass balance results showed that the TN removal in the FHSCW, SHSCW and SFCW was 17.08 g/m², 23.57 g/m² and 6.29 g/m², respectively. The TN removal efficiency in the HSCW was 2.7-3.7 times that in the SFCW. In the MCWP, the HSCW played a leading role in TN removal, which was mainly caused by different substrates. In the HSCW, the substrates were gravel and steel slag, while the substrate of the SFCW was soil. Compared with soil, gravel and steel slag have larger specific surface areas and stronger adsorption capacities (Lu et al., 2016) and are more conducive to the growth and reproduction of microorganisms (Sun et al., 2020). Therefore, the TN removal efficiency in the HSCW was better than that of the SFCW. The results showed that the substrate played an important role in the purification of polluted water by CWs.

Plant uptake played an important role in TN removal in the CWs. Based on previous research, the nitrogen content of *Iris pseudacorus* L. leaves was 20.11 mg/g. The TN uptake of *Iris pseudacorus* L. was 2.66 g/m² (FHSCW), 2.76 g/m² (SHSCW) and 3.43 g/m² (SFCW), accounting for 15.57 %, 11.71 % and 54.53 % of the total removal, respectively. In the CWs used for surface water pretreatment (i.e., the HSCW in this study), the amount of nutrients removed by harvesting plants was not significant. In the SFCW used for advanced treatment, harvesting plants played a greater role in nitrogen removal. The results of Vymazal et al. (1998) were consistent with those of this study, i.e., the amount of nitrogen removed by harvesting in CWs for secondary treatment accounted for 10 %-16 % of the total N removal under optimal conditions. The amount of nitrogen removed by harvesting in CWs for advanced treatment may play a more important role. Therefore, in the study of the SFCW, a series of problems urgently need to be solved, such as harvesting time, harvesting frequency, and the relationship between the release of pollutants and environmental factors in CWs, which are of great significance to improve the purification effect of the SFCW.

The purification effect of the SFCW directly depends on the retention time of pollutants (Sabokrouhiyeh et al., 2016). The HRT in the SFCW increased with increasing plant density (Jaddhav and Buchberger., 1995). The planting density was positively correlated with the HRT. Properly increasing planting density could improve mixing efficiency in CWs. When the planting density reaches a certain threshold, the HRT of CWs increases slowly (Sabokrouhiyeh et al., 2016). Luo (2005) showed that increasing planting density could improve the removal effect of $\text{NH}_4^+\text{-N}$ and TN. However, when the planting density was too high, further increasing planting density had little effect on the improvement of nitrogen removal, even though it was not conducive to nitrogen removal. Therefore, when the SFCW was used for advanced treatment, a suitable high-density planting method could effectively increase the proportion of nutrients removed by plant harvesting and enhance the level of pollutant removal.

Analysis of bacterial community structure and composition in CWs

Alpha diversity analysis

Table 3 Alpha diversity index of bacteria at the genus level in CWs.

Sample	Sobs	Shannon	Simpson	Ace	Chao	Coverage
FHSCW	621	5.028574	0.013884	676.940617	689.695652	0.998762
SHSCW	683	5.162082	0.013927	731.525148	731.766667	0.998734
SFCW	763	5.460566	0.008145	821.572446	822.333333	0.997769

The functions and behaviors of bacteria influence the performance of CWs. Therefore, the bacterial community diversities and phylogenetic structures were analyzed through Illumina pyrosequencing in the two-level HSCW and SFCW, which are the core units of the MCWP. **Table 3** summarizes the richness and diversity indices of the bacterial community at the genus level in the CWs when the system was operated on the 59th day. The alpha diversity indices were calculated based on operational taxonomic units (OTUs) at a 97 % cutoff, with 2491, 2612 and 2975 OTUs identified in the two-level HSCW and SFCW, respectively. The number of OTUs increased gradually in the CWs along the MCWP. The results showed that ACE, Shannon, Chao1 and Sobs were greater in the SFCW, and coverage and Simpson were smaller in the SFCW than in the HSCW. The ACE and Chao1 indices indicate bacterial community richness, and Sobs is the actual observation of richness. The Shannon and Simpson indices reflect bacterial community diversity (Lu et al., 2020). Therefore, compared with the HSCW results, the bacterial community richness and diversity were higher in the SFCW. This difference might be because of the radial oxygen loss and root exudates in the SFCW with high-density planting, which had a significant impact on rhizosphere bacterial activity, community richness, diversity and function (Chen et al., 2015; Liang et al., 2016; Chen et al., 2019). Plants might enhance nitrification efficiency by releasing oxygen from roots, supporting the growth of nitrifiers (Li et al., 2018). Denitrification could also be promoted by organic compounds in plant exudates, which provide a carbon source for denitrifiers (Hou et al., 2018). Therefore, plant roots create favorable environments for the growth and reproduction of specific bacterial communities in the rhizosphere, especially ammonia-oxidizing bacteria (AOB) and NOB (Faulwetter et al., 2013). It was found that AOB and NOB in the rhizosphere were 17.9 % and 26.8 % higher, respectively, than those in the non-rhizosphere by the most-probable-number method (Hua et al., 2017). Therefore, the SFCW showed greater bacterial diversity and was more effective in removing nitrogen from actual polluted river water.

Bacterial species composition analysis

As shown in **Figs. 14** and **15**, the main bacteria detected in the two-level HSCW and SFCW were *Saprospiraceae*, *Gemmatimonadaceae*, *Gammaproteobacteria* and *Alphaproteobacteria*. At the genus level, *Thiobacillus*, *Terrimonas*, *Hygromobium* and *Nitrospira* were the dominant bacteria in the two-level HSCW, and *Hydrogenophaga* was the dominant bacteria in the SFCW.

Proteobacteria is one of the main dominant bacteria in wastewater treatment plants. The change in the sludge bacterial community in the actual printing and dyeing of wastewater by biological treatment systems also shows that *Alphaproteobacteria* and *Gammaproteobacteria* are the main dominant bacteria (Yang, et al., 2012). *Gammaproteobacteria* can metabolize glucose to produce acid, reduce nitrate to nitrite, use ammonium as a nitrogen source and glucose as a carbon source, and grow at 10-43 °C. *Saprospiraceae* is the main bacteria family causing filamentous bulking of sewage treatment plant sludge. It is a strictly aerobic chemoorganotrophic bacterium whose optimum living environment temperature is 30-37 °C, and when the pH is approximately 7, it can metabolize glucose, galactose, acetate, etc. The nitrifying bacteria in the activated sludge of sewage treatment plants are mainly *Nitrospira*, which can oxidize nitrite into nitrate in CW media, and *Nitrospira* are the main nitrogen removal functional bacteria in the FHSCW. So far, *Nitrospira* have been reported in many papers. NOB are dominant in the process of biological nitrogen removal. As autotrophic bacteria, when coexisting with heterotrophic bacteria, they are easily restrained due to their long growth cycle and lack of competitive advantage in space and time. Therefore, the species abundance was low in the CWs of this study. *Gemmatimonadaceae* has strong resistance to extreme environments. *Thiobacillus* is a colorless, rod-shaped and facultative sulfur-oxidizing bacterium that mainly exists in sediment and wet soil. Among them, *Thiobacillus denitrificans* is the most common genus of *Thiobacillus* in sewage treatment and is a kind of autotrophic denitrifying bacteria. It can obtain energy through sulfide oxidation in a low carbon source environment and take nitrate as an electronic receptor to generate nitrogen, in which nitrite and nitric oxide accumulate as intermediate products. The *Hydrogenophaga* detected in the SFCW are heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria. In recent years, the discovery of HN-AD bacteria has altered the traditional theory that nitrification can be performed only by autotrophic bacteria and denitrification can be carried out only under anaerobic conditions. The theory of HN-AD has become a hot topic at home and abroad. The HN-AD bacteria isolated from the sediment of the seabed were identified as *Klebsiella* sp. It has been proven that the bacteria have high HN-AD efficiency (Sun et al., 2016). The bacteria selected from the activated sludge of landfill leachate were identified as *Pseudomonas*. The study showed that the bacteria had high nitrogen removal characteristics and could realize simultaneous nitrification and denitrification (Wang et al., 2014). Compared with traditional denitrifying bacteria, HN-AD bacteria have greater advantages in nitrogen removal and organic matter removal. The carbon source, C/N and DO are the main factors affecting nitrogen removal. The free surface of the SFCW promoted air reoxygenation, and the high-density planting mode enhanced radial oxygen loss and improved the aerobic

conditions for the survival of HN-AD bacteria. Therefore, it is of great significance to study the natural restoration of polluted water by HN-AD bacteria.

Bacterial species diversity analysis

According to the bacterial community richness data obtained, a significance test of intergroup differences was carried out. Bacterial species with different abundances in different wetland units (samples) were detected by strict statistical methods, and the significance of the observed differences was evaluated by hypothesis tests. Fisher's exact test and FDR multiple test were selected as test methods.

As shown in **Fig. 16**, *Saprospiraceae*, the dominant species in the FHSCW, increased significantly in the SHSCW ($P < 0.01$). This finding was consistent with our previous research (i.e., among units, the SHSCW has the best COD_{Mn} removal effect, data not presented in this study). *Saprospiraceae* are strictly aerobic chemoorganotrophic bacteria. The large specific surface area of the gravel and steel slag in the HSCW provides a place for the growth and reproduction of bacteria. The genus *Terrimonas* is a known type of bacteria that can undergo aerobic denitrification. Its species abundance decreased significantly in the SHSCW because when the DO in the influent was consumed by the FHSCW and then pushed into the SHSCW, aerobic chemoorganotrophic bacteria, such as *Saprospiraceae*, further consumed the DO and organic matter in the water, which was not conducive to the growth and reproduction of *Terrimonas*. It was also found that the relative abundance of autotrophic bacteria such as *Thiobacillus* and *Nitrospira* decreased significantly, which may have been due to the survival competition of heterotrophic spirochetes.

As shown in **Fig. 17**, the species abundance of *Saprospiraceae*, the dominant species in the HSCW, was significantly decreased in the SFCW because the COD concentration in the effluent after the raw water flowed through the two-level HSCW was reduced to the lowest level of the system (data not presented in this study), which also limited the growth of heterotrophic bacteria. *Hydrogenophaga* are HN-AD bacteria, and the species abundance of these new denitrifying functional bacteria increased significantly in the SFCW, which indicated that the root exudates and radial oxygen loss in the SFCW with high-density planting improved the rhizosphere microenvironment. At the same time, the species richness of *Alphaproteobacteria* and *Sphingomonas* increased significantly in the SFCW. *Alphaproteobacteria* (root nodule bacteria) have been shown to be capable of denitrification under autotrophic and heterotrophic conditions in liquid media (Vilar-Sanz et al., 2018). *Sphingomonas* widely exists in plant rhizosphere soil and has good biological fixation of nitrogen and biodegradation functions (Hansen et al., 2020; Ali et al., 2019). A *Sphingomonas* sp. strain isolated and screened from a shrimp culture pond efficiently removed both ammonia nitrogen and nitrite (Yun et al., 2019). Therefore, the bacteria completed the nitrogen cycle in the SFCW with high-density planting through a variety of nitrogen removal pathways.

Conclusions

An MCWP was constructed to investigate the influence of seasonal fluctuations in temperature on nitrogen removal in the system. It was found that the TN concentration in the MCWP gradually decreased. The average removal efficiency in the MCWP was approximately 40.74%. The removal efficiency in the SPP was the best among all units, with a value up to 16.08%. It was found that the TN total removal efficiency was significantly positively affected by the daily high temperature. A formula between the total removal efficiency and the highest temperature was obtained by nonlinear fitting, and the fitting result R^2 reached 0.7192. The TN removal load rate per unit area in the HSCW was 2.7–3.7 times that in the SFCW. The bacteria completed the nitrogen cycle in the SFCW, which had high-density planting (35 plants/m²), through a variety of nitrogen removal pathways. Furthermore, the TN uptake by *Iris pseudacorus* L. accounted for 54.53% of the total TN removal in the SFCW. The SFCW, as an advanced treatment unit, could effectively increase the proportion of nutrients removed by plant harvesting and enhance the removal level of pollutants.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Contributions

Lu H B conceived and designed the experiments. Wang T, Li J X, Xiao L P and Zhao X L performed the experiments. Wang T analyzed and interpreted the data. Wang T and Lu H B were major contributors in writing the manuscript. Lu S Y and Guo X C funded the work.

Acknowledgments

This work was financially supported by Ministry of Science and Technology of China (Grant No.2015FY110900), the National Natural Science Foundation of China (41877409) and the Major Science and Technology Program for Water Pollution Control and Treatment of China (2018ZX07208008).

Funding

None.

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Figures

Figure 1

System process flow plan

Figure 2

Schematic diagrams: (a) HSCW; (b) SFCW

Figure 3

TN removal effect in EP

Figure 4

TN removal effect in OP

Figure 5

TN removal effect in FHSCW

Figure 6

TN removal effect in SHSCW

Figure 7

TN removal effect in SFCW

Figure 8

TN removal effect in SPP

Figure 9

Total TN removal effect in the MCWP

Figure 10

NH₄⁺-N removal effect in the MCWP: (a) concentration curve; (b) removal rate curve

Figure 11

Changes in plant height in the MCWP

Figure 12

TN/NH₄⁺-N total removal rate and temperature change with time

Figure 13

Fitting curve of the TN total removal rate and lowest temperature

Community heatmap analysis on Genus level

Figure 14

Heatmap of bacterial community at the genus level

Community barplot analysis

Figure 15

Bar chart of bacterial community at the genus level

Fisher's exact test bar plot on Genus level

Figure 16

Bacterial species diversity analysis of the two-level HSCW

Figure 17

Bacterial species diversity analysis of the SHSCW and SFCW