

Performance and microbial community in a combined VF-HF system for the advanced treatment of secondary effluent

Zhichao Zhu, Hengfeng Miao, Yajing Zhang, Jian Cui, Zhenxing Huang and Wenquan Ruan

ABSTRACT

In this study, a laboratory-scale system combined a vertical flow constructed wetland (VF) with a horizontal flow constructed wetland (HF), which was used to treat the secondary effluent of a wastewater treatment plant. Removal efficiencies of 67.02%, 89.80%, 90.31% and 75.38% were achieved by the system for chemical oxygen demand (COD), ammonium nitrogen ($\text{NH}_4^+\text{-N}$), total nitrogen (TN) and total phosphorus (TP), respectively. The VF showed much higher average loading rates of COD, TP, $\text{NH}_4^+\text{-N}$ and TN (7.96 g/m²/d, 0.076 g/m²/d, 0.31 g/m²/d and 0.99 g/m²/d) than in HF (0.65 g/m²/d, 0.016 g/m²/d, 0.25 g/m²/d and 0.50 g/m²/d), during the stable operation period. Biodegradation played a major role in pollutant removal, especially for COD and TN. The results of bacterial community analysis indicated that heterotrophic denitrifying bacteria (*Hydrogenophaga* and *Flavobacterium*) were the dominant contributors for nitrogen removal in the VF, while heterotrophic denitrifying bacteria (*Rhodobacter*, *Flavobacterium* and *Dechloromonas*) and the autotrophic denitrifying bacteria *Sulfurimonas* played the principal roles for nitrogen removal in the HF. Redundancy analyses showed that COD and $\text{NH}_4^+\text{-N}$ were the important factors affecting the distribution of nitrogen removal bacteria in the VF, while pH, dissolved oxygen and oxidation-reduction potential were the key factors influencing the distribution of nitrogen removal bacteria in the HF.

Key words | autotrophic denitrification, heterotrophic denitrification, redundancy analysis, secondary effluent, VF-HF system

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INTRODUCTION

The secondary effluents from wastewater treatment plants (WWTPs) with relatively high nutrients can lead to water quality deterioration and eutrophication in those receiving water bodies (Zhou *et al.* 2011). In particular, the total nitrogen (TN) concentration in the secondary effluent is commonly in the range of 12.0–15.0 mg/L (Gao *et al.* 2017a), which is much higher than the surface-water criteria of the Chinese National Surface Water Environmental Quality Standard (2.0 mg/L for grade V, GB3838-2002, 2002). Moreover, surface-water contamination is regarded as a serious problem in China, with 11.5% severely polluted freshwater and 26.2% slightly polluted freshwater in major lakes and reservoirs (Gao *et al.* 2017a). Hence, advanced treatment of secondary effluent is of great importance, especially for TN removal.

Advanced tertiary treatments for secondary effluents from WWTPs have been developed, such as activated carbon adsorption (Pramanik *et al.* 2015) and membrane-based technologies (Yang *et al.* 2015). However, these advanced tertiary treatment technologies require a substantial outlay and high operating and maintenance costs. By comparison, constructed wetlands (CWs) as an ecologically efficient solution seem to be more feasible for secondary effluent treatment, with advantages in terms of low cost, easy operation and maintenance as well as limited secondary pollution. Two types of CWs, classified according to water flow–vertical flow constructed wetland (VF) and horizontal flow constructed wetland (HF) – are extensively used. A combined vertical flow and horizontal flow constructed wetland (VF-HF) system includes the advantages of two

types of CWs to provide appropriate aerobic and anaerobic conditions for organic matter and TN removal (Vymazal & Kröpfelová 2011, 2015). Secondary effluent is characterized by high nutrients, high refractory organic matter and varied water quality and quantity, which are difficult to treat effectively in single CWs (Xu *et al.* 2016). Therefore, the multi-stage system of VF and HF was developed for the purpose of pollutant removal from secondary effluent to produce better quality effluent (Xu *et al.* 2016).

Previous investigations have shown that pollutants in secondary effluent, such as chemical oxygen demand (COD), TN, ammonia nitrogen ($\text{NH}_4^+\text{-N}$) and total phosphorus (TP), can be removed by physical, biochemical and the combination of physical and biochemical processes. Microbial activities in biochemical processes in the CWs are recognized as a major contributor to the removal of pollutants in wastewater, especially for nitrogen (Wu *et al.* 2016). However, microbial community structure and their metabolic activities can be easily affected by changing operation conditions such as pH, dissolved oxygen (DO), temperature, carbon source and nitrogen oxides, etc. (Wu *et al.* 2016; Xu *et al.* 2016).

Therefore, a laboratory-scale combined VF-HF system was developed for secondary effluent treatment. The main objectives of this investigation were to (1) evaluate the contaminant removal efficiencies of the VF-HF system for secondary effluent treatment of WWTPs, (2) explore the microbial community distribution in the system, and (3) gain insight into the relationships between the active microbial populations involved in nitrogen removal with the associated environmental variables.

MATERIAL AND METHODS

Experimental system and conditions

The laboratory-scale combined VF-HF system was located at Jiangnan University ($120^\circ 27' 39.20''\text{E}$, $31^\circ 49' 02.51''\text{N}$) in Wuxi City (China), where a subtropical monsoon climate predominates. The system consisted of a regulating tank, followed by a VF unit and an HF unit in series, as is shown in Figure S1 (available with the online version of this paper). The regulating tank (500 L) was used for the homogenization of the influent, which was the secondary effluent of a WWTP in Wuxi City. The VF was a cylinder (Φ 60 cm \times 100 cm) made of stainless steel, with an effective volume of 280 L. From bottom to top, the VF was filled with 30 cm gravel with particle size 10–30 mm, 30 cm active

carbon with particle size 5–10 mm, and 30 cm zeolite with particle size 4–5 mm. The HF was a rectangular tank (100 cm \times 50 cm \times 50 cm) also made of stainless steel, with an effective volume of 250 L. The HF was filled with 20 cm zeolite with particle size 4–5 mm as the bottom layer, and 20 cm quartz sand with particle size 2–4 mm as the upper layer.

The secondary effluent of a nearby WWTP with inverted A^2/O process was pumped into the regulating tank, then was further continuously guided into the VF-HF system, with a daily treatment capacity of 60 L. In this process, the hydraulic loading rates of the VF and HF were calculated as 21.23 cm/day and 12.00 cm/day, respectively. The water quality of the secondary effluent is shown in Table S1 (available online). The average concentrations of COD, $\text{NH}_4^+\text{-N}$, TN and TP were 62.85 mg/L, 3.86 mg/L, 9.70 mg/L and 0.63 mg/L, respectively, with the COD/TN ratio of 6.48.

Sampling strategy and monitoring

Water samples from each treatment unit such as the effluents of the regulating tank, the effluents of the VF and the effluents of the HF were collected every two days. Water quality parameters such as pH value, DO concentration and oxidation-reduction potential (ORP) value were determined onsite at the time of sample collection. Water samples were transferred to the laboratory for immediate measurement of the following parameters: COD, $\text{NH}_4^+\text{-N}$, nitrate nitrogen ($\text{NO}_3^-\text{-N}$), TN and TP. All the water samples were collected in triplicate.

Additionally, in order to investigate the complete microbial community structures, microbial samples were collected from the water and biofilm on substrates according to Adrados' method (Adrados *et al.* 2014), at the end of the system operation. Five microbial samples were collected from the water of secondary effluent (WSE) in the regulating tank, the top layer of the VF (VF1-1, 0–30 cm), the bottom layer of the VF (VF1-2, 60–90 cm), the top layer of the HF (HF1-1, 0–20 cm) and the bottom layer of the HF (HF1-2, 20–40 cm), respectively. All the microbial samples were collected in triplicate. The sampling areas are shown in Figure S1.

Water sample analysis

The pH, DO and ORP values of the water samples were immediately measured with a portable multi-parameter water quality analyzer (PRO1020, YSI, USA). After the corresponding standard pretreatment and reagent addition, the

concentrations of COD, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, TN and TP were determined according to *Standard Methods* (APHA 2005).

Microbial community analysis

Total bacterial DNA was extracted from a 250 mg pellet of the above-mentioned samples using MP FastDNA[®] SPIN kit (MP Biomedicals, USA), according to the manufacturer's protocol. The concentration and purity of DNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA), and the integrity of DNA was evaluated via 1% agarose gel electrophoresis (5 V/cm, 20 min) before it was used for amplification. The V3-V4 region of bacterial 16S rRNA genes was amplified using the universal primers 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') according to Xu's method (Xu *et al.* 2016). A mixture of the amplicons was then used for sequencing on the Illumina PE300 at the Genomic Research Center at Shanghai Majorbio Biopharm Biotechnology Co. Ltd, Shanghai, China (Wang *et al.* 2016).

Statistical analysis

The rarefaction curve and clustering analyses were plotted with R tools. The relationships between microbial community structures and the environmental variables were analyzed using redundancy analysis based on R vegan package. The Monte Carlo permutation test was carried out to ascertain the significance of the relationships between

microbial community structures and the selected environmental variables.

RESULTS AND DISCUSSION

Overall performance of organic matter and nutrients removal

The advanced treatment of secondary effluent from a WWTP was investigated in the VF-HF system in this investigation, for the purpose of organic matter and nutrient removal. After ~44 days of operation, the system became stable with the COD, $\text{NH}_4^+\text{-N}$, TN and TP concentrations of the system effluents below 30.00 mg/L, 1.00 mg/L, 1.00 mg/L and 0.30 mg/L, respectively (Figures 1 and 2). This meant that the effluents of VF-HF system could reach the 'Surface Water Environmental Quality Standard' (GB3838-2002) grade IV, in most cases. Specifically, removal characteristics of COD, TP and TN in the system were explained as follows.

COD removal

Figure 1(a) shows COD concentrations of the influents, VF effluents and HF effluents respectively, during the whole operation period. In the first 10 days of operation, the VF-HF system showed a high average COD removal efficiency of 73.11%, and COD was observed to be mainly removed in the VF. This is most likely due to the adsorption and retention by the substrates such as zeolite and active carbon in the VF (Zhou *et al.* 2017). Then, COD

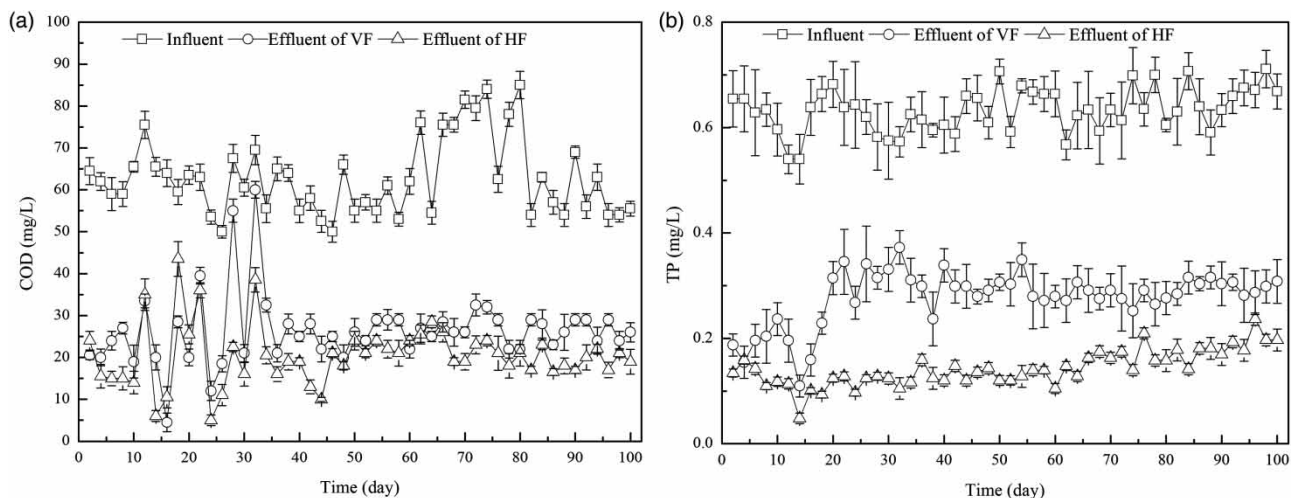


Figure 1 | COD and TP concentration variations during the VF-HF system operation: (a) COD; (b) TP.

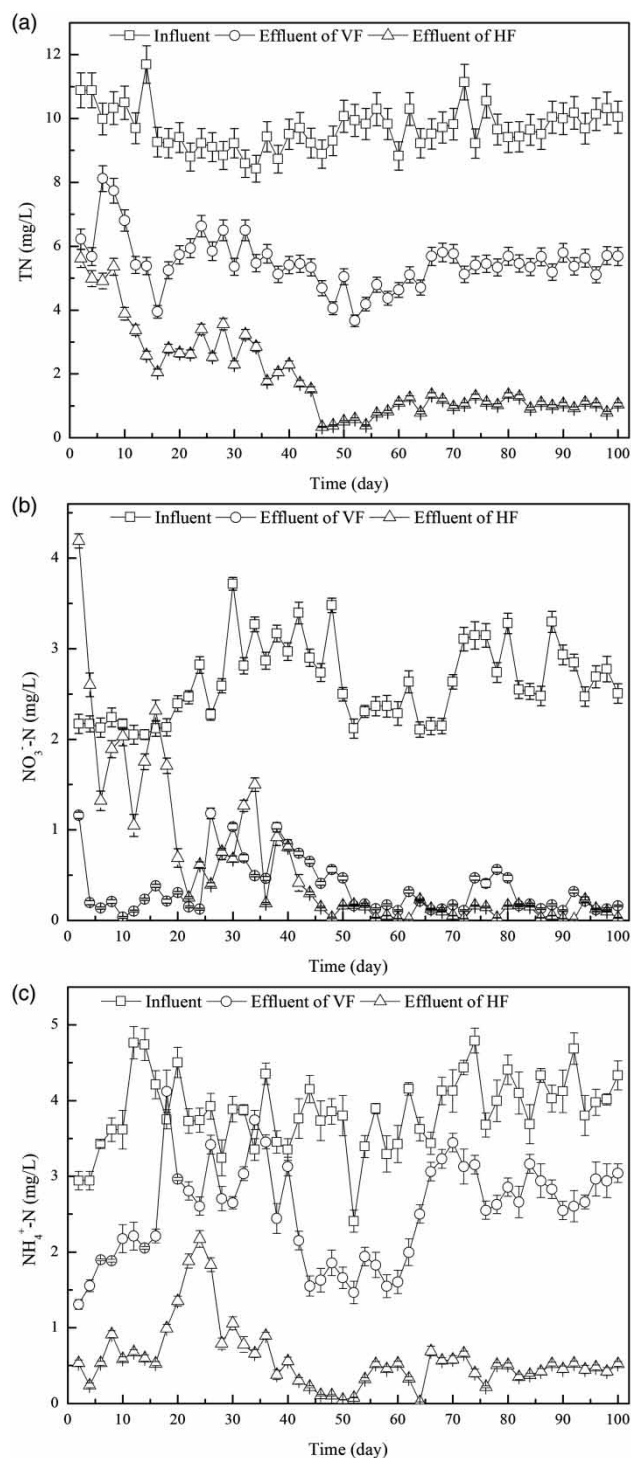


Figure 2 | TN, NO₃⁻-N and NH₄⁺-N concentration variations during the VF-HF system operation: (a) TN; (b) NO₃⁻-N; (c) NH₄⁺-N.

removal efficiency fluctuated during the prolonged operation time from the 12th day to the 32nd day, which ranged from 26.89% to 90.84%. The decrease of COD removal efficiency may be due to the saturation of

substrates' adsorption capacity (Zhou *et al.* 2017). After 44 days of operation, the treatment performance of the VF-HF system was observed to be stable and biodegradation was presumed to play a dominant role within the system. The system exhibited an overall COD removal efficiency of 67.02% after 44 days, which was higher than Xu *et al.* (2016) (up to about 55%) under similar climatic conditions. Specifically, 85.58% COD removal occurred in the VF. For example, during this period, the average COD concentrations of the influents, VF effluents and HF effluents were 63.95 mg/L, 26.46 mg/L and 21.09 mg/L, respectively. As shown in Table S2 (available online), the average COD loading rate in the VF (7.96 g/m²/d) was higher than in the HF (0.65 g/m²/d) during the stable operation period. This indicated that the substrates in the VF provided an appropriate place for microbial growth, and thus strengthened COD degradation (Zhou *et al.* 2017).

Phosphorus removal

The results presented in Figure 1(b) indicate that both the VF and the HF contributed to phosphorus removal. After 44 days of operation, the treatment performance of the VF-HF system was observed to be stable. Mean TP concentrations of influents, and effluents in the VF and the HF were 0.65 mg/L, 0.29 mg/L and 0.16 mg/L, respectively. The VF contributed 55.38% TP removal efficiency individually, and the VF-HF system revealed a 75.38% TP removal efficiency overall. The removal efficiency of TP was similar to Xu *et al.* (2016) (77.8%). As shown in Table S2, the average TP loading rate in the VF (0.076 g/m²/d) was higher than in the HF (0.016 g/m²/d) during the stable operation period. This suggested that most TP was intercepted in the VF, by the removal pathway of substrates (e.g. active carbon, zeolite) adsorption (Dong *et al.* 2012).

Nitrogen removal

Figure 2 illustrates the nitrogen removal in the VF-HF system during the operation period. As shown in Figure 2(a), TN removal efficiency increased with the increase of operation time. The overall removal efficiency of TN rose from 48.39% to 83.83% until the 44th day, then further increased to 90.31% at the end of this process (after 44 days), which was higher than that previously reported by Ong *et al.* (2009) (67%) and Xu *et al.* (2016) (69.8%). In addition, NO₃⁻-N as the denitrification electron acceptor also showed a similar removal tendency to TN removal, which is shown in Figure 2(b). Results indicated that the

microorganisms of nitrification and denitrification played important roles in the VF-HF system for TN removal (Xu *et al.* 2016). The enrichment and evolution of the bacterial communities continuously developed with the prolonged operation time.

Comparing TN removal in the VF with those in the HF after 44 days, the HF (81.59%) showed much a higher TN removal efficiency than the VF (47.35%). However, from the perspective of loading rates, the average TN loading rate in the VF (0.99 g/m²/d) was higher than in the HF (0.50 g/m²/d) during the stable operation period, especially after 44 days (Table S2). This result was consistent with Tao *et al.* (2017), in which NO₃⁻-N concentrations in the effluents of the VF and the HF were relatively low with the mean value of 0.24 mg/L and 0.10 mg/L respectively (Figure 2(b)), demonstrating sufficient denitrification in both units.

The NH₄⁺-N removal in the VF-HF system is shown in Figure 2(c). At the beginning of the operation, NH₄⁺-N concentrations of the VF effluents and HF effluents increased with the increase of operation time. The NH₄⁺-N concentrations in the VF and HF effluents reached the maximum values of 4.12 mg/L and 2.17 mg/L on the 18th day and the 24th day, respectively. At this point, the microorganisms of nitrogen removal were not well evolved, and NH₄⁺-N was presumed to be chiefly removed by substrate adsorption (Lu *et al.* 2016; Zhou *et al.* 2017). The decrease of NH₄⁺-N removal efficiency might be explained by the saturation of the substrates with the prolonged operation time (Lu *et al.* 2016; Zhou *et al.* 2017). With the accumulation and evolution of nitrogen removal microorganisms, NH₄⁺-N removal efficiency increased, especially in the HF. During the stable period (after 44 days), the average NH₄⁺-N concentrations of VF effluents and HF effluents were 2.51 mg/L and 0.40 mg/L, respectively, showing a 84.06% NH₄⁺-N removal rate in the HF and 89.80% in the whole system. The NH₄⁺-N removal efficiency in this system was slightly higher than previously reported by Xu *et al.* (2016) (82.3%). Moreover, as shown in Table S2, the NH₄⁺-N loading rate (0.31 g/m²/d) in the VF was higher than in the HF (0.25 g/m²/d) during the operation period. This result was consistent with Tao *et al.* (2017). The VF was principally operated in anaerobic-anoxic conditions, with the average DO concentrations in the top layer of 0.74 mg/L and in the bottom layer of 0.13 mg/L, respectively (Figure S2, available with the online version of this paper). Low NH₄⁺-N removal efficiency in the VF (Figure 2(c)) might be caused by nitrification inhibition with the low DO concentration and ammonification from the organic nitrogen (Vymazal & Kröpfelová 2011; Xia *et al.* 2013). The HF provided a

favorable aerobic environment for NH₄⁺-N nitrification, especially in the upper layer of the HF (DO concentration 3.79 mg/L). Moreover, the anaerobic-anoxic conditions in the bottom of the HF favored denitrification (DO concentration 0.60 mg/L), which led to the high TN removal efficiency. In addition, relatively high organic nitrogen (~3.22 mg/L) in the influents also could be transformed into NH₄⁺-N through anaerobic ammonification by *Pseudomonas* (Li *et al.* 2008). This also might be the main reason for the increase of NH₄⁺-N concentrations in the effluents (Xia *et al.* 2013) of VF from the 62nd day to the 66th day.

Microbial community

Previous studies have shown that nitrogen is mainly removed by microorganisms in CWs by nitrification-denitrification (Zhong *et al.* 2015; Wu *et al.* 2016). Results in the 'Nitrogen removal' section also demonstrated that biodegradation played an important role in the VF-HF system. Hence, bacterial communities in the VF and HF were characterized by using 16S rRNA high-through sequencing. Rarefaction analysis was used to standardize and compare the reflected microbial diversity among the samples, recognizing the rationality of samples sequencing data. For the Sobs index (Figure S3(a)), the rarefaction curves approached a plateau, suggesting that further sequencing will result in few additional operational taxonomic units (OTUs). In addition, rarefaction curves in the Shannon index (Figure S3(b)) reached a plateau, indicating that sequences reflect the majority of microbial diversity information among the samples. Therefore, results in Figure S3 verified that the sequencing data are reasonable. (Figure S3 is available online.)

Diversity and clustering analysis of the microbial community

As is presented in Table S3 (available online), 20,432–26,932 OTUs were observed in the five samples, demonstrating that the microbial communities were highly complex in each area of the VF-HF system. Community diversity (Shannon, Simpson), richness (Sobs, ACE, Chao 1) and calculated coverage percentage (Good's coverage) of the samples are also listed in Table S3. Good's coverage indices clustered from the OTUs of the samples were all higher than 0.99, further suggesting that the collected gene sequences could represent the bacterial OTUs in each sample. Results of the indices indicated that the microbial community in the WSE had lower richness and diversity than in the VF-HF

system. In addition, the samples from the bottom layers (Sobs 1,451–1,502, ACE 1,767–1,775, Chao 1 1,823–1,826, Shannon 5.77–5.86 and Simpson 0.01–0.01) had obviously higher richness and diversity than those from the top layers (Sobs 819–821, ACE 981–1,051, Chao 1 993–1,044, Shannon 4.87–5.01 and Simpson 0.02–0.03), which showed no obvious difference in the different units.

Hierarchical clustering analysis (Figure S4, available online) suggested WSE, VF1-1 and HF1-1 were in one group, and VF1-2 and HF1-2 consisted of the other group. This meant a significant difference in the microbial community structure between the top and bottom layers in the same units. Results were similar to the result from indices analysis. Environmental factors such as DO distribution (Figure S2) in the units might have contributed to shaping the bacterial community (Wu et al. 2016; Xu et al. 2016).

Classification and identification of the microbial community

In addition to the richness and diversity of the microbial communities, the community composition in the CWs system played critical roles in pollutant removal (Xu et al. 2016). In order to compare differences in the compositions of the microbial community, relative community abundance of the five samples in VF-HF system were characterized at the phylum, class, family and genus levels.

Fourteen major bacteria (relative abundance >1%) at the phylum level are shown in Figure 3(a). *Proteobacteria* and *Bacteroidetes* were the top two predominant phyla in the area of the WSE, VF1-1 and HF1-1, accounting for 82.49%, 90.94% and 82.08% of the total microbial communities, respectively. The relative abundance of *Firmicutes* and *Spirochaetae* in the WSE were 5.04% and 4.16%, respectively. *Actinobacteria* and *Cyanobacteria* also contributed the relatively low proportion of 3.71% and 3.54% to the total microbial communities respectively, in HF1-1. As for those in VF1-2 and HF1-2, *Proteobacteria*, *Bacteroidetes* and *Parcubacteria* were the top three predominant phyla, in total accounting for 64.99% and 68.84% of the microbial communities, respectively. Other bacteria such as *Actinobacteria* (2.61–5.17%), *Cyanobacteria* (2.33–5.61%), *Gracilibacteria* (3.99–5.61%), *Firmicutes* (1.26–5.37%) and *Chloroflexi* (1.93–2.66%) showed much lower relative abundance in the area of VF1-2 and HF1-2. As should be pointed out, most of the bacteria involved in nitrification and denitrification belong to *Proteobacteria* (Gao et al. 2017b). *Bacteroidetes* and *Chloroflexi* are mostly denitrifying bacteria (Jin et al. 2014). This meant a great potential for

nitrogen removal bacteria in the VF-HF system. In addition, substantial differences in relative abundance were observed in the phylum distribution of the five samples. From the secondary effluent (WSE) to the VF-HF system (VF1-1), the relative abundance of *Proteobacteria* and *Bacteroidetes* decreased slightly, and the relative abundance of *Firmicutes* and *Spirochaetae* decreased significantly. *Parcubacteria*, *Gracilibacteria* and *Firmicutes*, with relative abundances of 13.32–15.86%, 3.99–5.61% and 1.26–5.37% respectively were more abundant in VF1-2 and HF1-2 than in VF1-1 and HF1-1 (less than 0.5%). *Nitrospirae*, proven to have the ability to perform nitrification by Zhong et al. (2015), showed a much higher relative abundance of 1.59% in HF1-1 than the other samples. According to DO concentrations in the VF-HF system (Figure S2), HF1-1 was primarily under aerobic conditions, while VF1-1, VF1-2 and HF1-2 were mainly under anaerobic and anoxic conditions. Microorganisms such as *Parcubacteria* and *Gracilibacteria*, which are suitable for growth in anaerobic and anoxic environments, might be involved in degradation of organic compounds and denitrification. *Nitrospirae* suggested high nitrification activities in HF1-1, and led to the high NH_4^+ -N removal efficiency (84.27% in Figure 2(c)) in HF.

At the class level, 25 major bacteria (relative abundance >1%) are shown in Figure 3(b). *Betaproteobacteria* (32.73%), *Gammaproteobacteria* (17.46%) and *Flavobacteriia* (16.41%) were the top three predominant classes in the WSE. *Betaproteobacteria* (32.73%), *Sphingobacteriia* (14.46%), and *Deltaproteobacteria* (14.05%) were the top three predominant classes in VF1-1. From the secondary effluent (WSE) to the VF-HF system (VF1-1), the relative abundance of *Betaproteobacteria*, *Sphingobacteriia* and *Deltaproteobacteria* increased, and the relative abundance of *Gammaproteobacteria* and *Flavobacteriia* decreased. *Sphingobacteriia* (16.40%), *Betaproteobacteria* (15.03%) and *Parcubacteria* (12.82%) were the top three predominant classes in VF1-2. This indicated that different areas in the VF had obviously different distribution of microbial classes. Besides, *Alphaproteobacteria* (29.77%), *Gammaproteobacteria* (21.40%) and *Sphingobacteriia* (13.74%) were the top three predominant classes in HF1-1. *Betaproteobacteria* (21.79%), *Parcubacteria* (11.03%) and *Sphingobacteriia* (8.50%) were the top three predominant classes in HF1-2. This further showed that there were obvious bacterial community differences in the different units and different areas in the HF. In particular, the denitrifying bacteria in the two samples of the HF, such as *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria* with the total relative abundance of 95.30%,

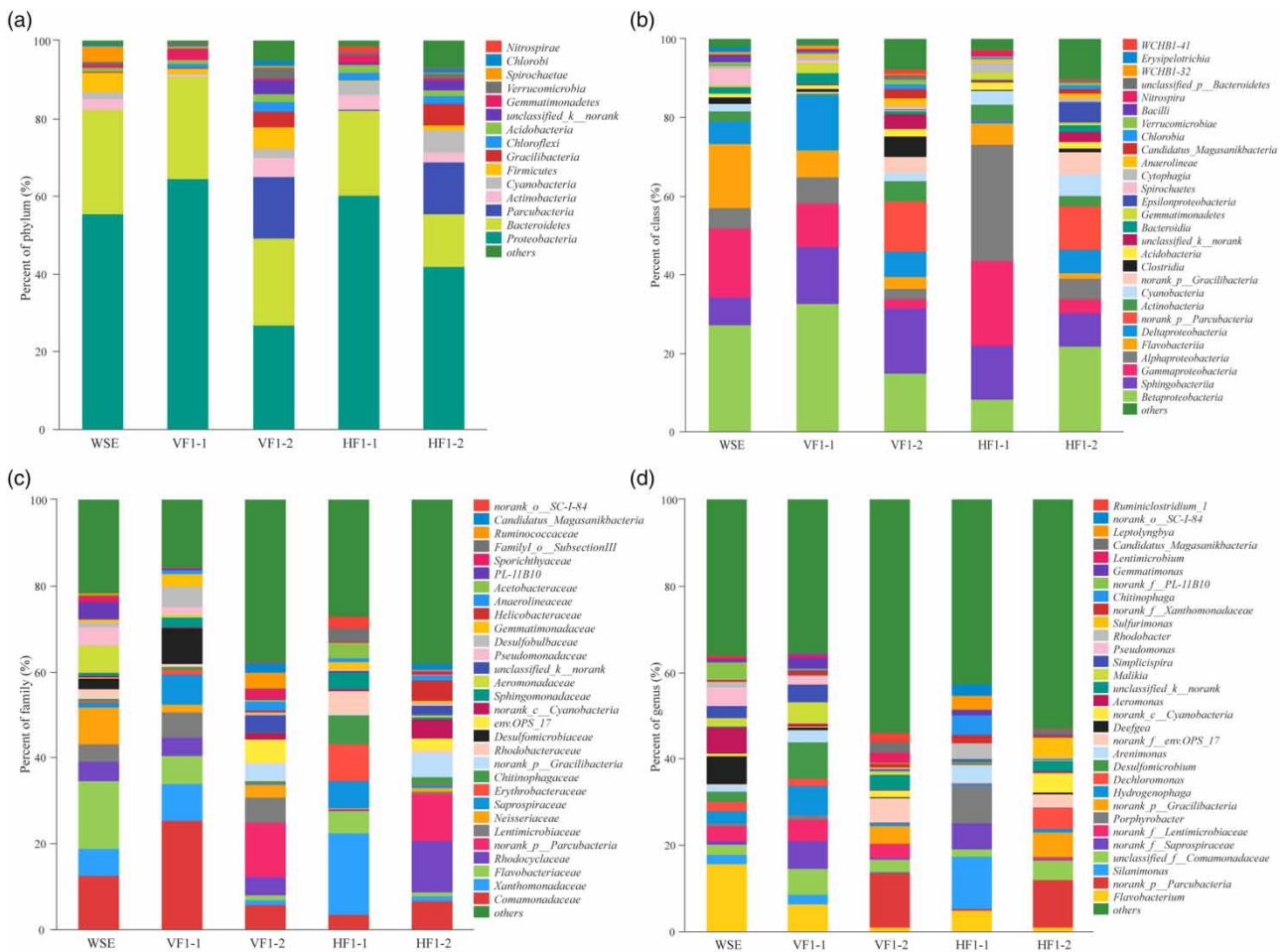


Figure 3 | Composition of the bacterial community at the phylum level (a), the class level (b), the family level (c), and the genus level (d) collected from the different sampling areas (WSE, VF1-1, VF1-2, HF1-1 and HF1-2) in the VF-HF system.

were much higher than in the VF (70.68%). *Betaproteobacteria*, a typical heterotrophic denitrifying bacteria, was observed to be accumulated in the areas of VF1-1 and HF1-2, and showed less abundance in the areas of VF1-2 and HF1-1. *Gammaproteobacteria*, a typical autotrophic denitrifying bacteria, had mostly accumulated in HF1-1 (21.40%), *Epsilonproteobacteria*, another typical autotrophic denitrifying bacteria, had mostly accumulated in the areas of HF1-2. Results were well consistent with those in Figure 2 (a). High TN removal efficiency in the HF of 81.59% might be due to the high relative abundance of denitrifying bacteria in the HF. *Nitrospira*, a typical nitrifying bacteria, was clearly detected in HF1-1, with the highest relative abundance of 1.59%, demonstrating higher nitrification in this area. This also could explain the fact that the HF had a higher $\text{NH}_4^+\text{-N}$ removal efficiency than the VF (Figure 2(c)).

At the family level, the major bacteria (relative abundance >2%) are shown in Figure 3(c). *Flavobacteriaceae* (15.78%),

Comamonadaceae (12.54%) and *Neisseriaceae* (8.56%) were the top three predominant families in the WSE. *Comamonadaceae* (25.54%), *Xanthomonadaceae* (8.47%) and *Desulfomicrobiaceae* (8.32%) were the top three predominant families in VF1-1. From the secondary effluent (WSE) to the VF-HF system (VF1-1), the relative abundance of the major bacteria (*Comamonadaceae*, *Desulfomicrobiaceae* and *Xanthomonadaceae*) increased, and the relative abundance of the major bacteria (*Flavobacteriaceae* and *Neisseriaceae*) decreased. *Comamonadaceae*, a possible heterotrophic denitrifying bacteria (Zhong et al. 2015), was the top predominant family in VF1-1 (25.54%), and was less abundant in HF1-2 (6.78%), VF1-2 (5.89%) and HF1-1 (3.65%). Another possible heterotrophic denitrifying bacteria, *Rhodocyclaceae*, was also observed in all areas of the VF-HF system, with the highest accumulation in HF1-2 (11.84%). The *Xanthomonadaceae* family (belonging to *Gammaproteobacteria*) were possible facultative autotrophic

denitrifying bacteria (Gao et al. 2017b). The relative abundance of *Xanthomonadaceae* was 18.89% in HF1-1 and 8.47% in VF1-1, respectively. In all, TN removal processes appeared to occur in the whole VF-HF system. *Comamonadaceae* was presumed to be the main microbial family for denitrification in the VF, and *Xanthomonadaceae* as well as *Rhodocyclaceae* were the dominant microbial families for denitrification in the HF.

At the genus level, the major bacteria (relative abundance >2%) are shown in Figure 3(d). *Flavobacterium* (15.76%), *Deefgea* (6.43%) and *Aeromonas* (6.16%) were the major bacteria in the WSE. From the secondary effluent (WSE) to the VF-HF system (VF1-1), the relative abundance of the major bacteria (*Flavobacterium*, *Deefgea* and *Aeromonas*) decreased, and the *Desulfomicrobium*, *Hydrogenophaga*, *Saprospiraceae*, etc. were enriched in VF1-1. Members of *Parcubacteria*, *Silanimonas*, *Saprospiraceae*, *Flavobacterium*, *Porphyrobacter* and *Desulfomicrobium* were most frequently detected. *Parcubacteria* showed higher abundance in both VF1-2 (12.82%) and HF1-2 (11.03%), and much lower abundance in VF1-1 (0.02%) and HF1-1 (0.33%) respectively. *Silanimonas* was the most abundant in HF1-1 (11.99%), and *Desulfomicrobium* was the most abundant in VF1-1 (8.32%). Overall, different dominant bacteria were distributed in the different CWs and functional areas, showing various mechanisms for pollution removal.

Major function genera for nitrogen removal

In previous studies, the genera of *Dechloromonas*, *Comamonas*, *Thiobacillus*, *Nitrosospira*, *Flavobacterium*, *Hydrogenophaga* and *Azospira* were considered to play a vital function in the removal of organic matter and nitrogen (Zhong et al. 2015; Wang et al. 2017). The major possible function genera involved in nitrogen removal in the four samples of the VF-HF system (VF1-1, VF1-2, HF1-1 and HF1-2) are summarized in Table 1. Nitrification consisted of two processes: the conversion of ammonium to nitrite by ammonium oxidizing bacteria (AOB) and the conversion of nitrite to nitrate by nitrite oxidizing bacteria (NOB). *Nitrosomonas*, belonging to AOB, had relatively high affinity constants for ammonia and relatively high maximum activity, which was detected in all the areas in the system (Domingos et al. 2011). The sequences belonging to *Nitrosomonas* in the HF were higher than in the VF. Hence, the diversity of *Nitrosomonas* in the HF was higher than in the VF. This indicated that nitrification resistance against perturbation in the HF was higher than that in the VF (Domingos et al. 2011). *Nitrosospira*, belonging to NOB, showed much higher

sequences in the HF than in the VF. This also indicated that nitrification resistance against perturbation in the HF was higher than that in the VF (Domingos et al. 2011). The results further demonstrated the high $\text{NH}_4^+\text{-N}$ removal efficiency in the HF.

The majority of denitrifying bacteria were enriched in the facultative environment, and utilized the organic compounds as electron donors for nitrogen removal. Table 1 lists the dominant genera which had been characterized as denitrifying bacteria (Zhong et al. 2015; Gao et al. 2017b; Wang et al. 2017). Although the sequences of the genera cannot represent all the denitrifying bacteria, the data were still helpful for revealing denitrifying bacteria distribution in the four sampling areas. It is well known that a large proportion of possible heterotrophic denitrifying bacteria belongs to *Beta-proteobacteria*, such as *Comamonadaceae* family (the genera of *Hydrogenophaga* and *Acidovorax*) and *Rhodocyclaceae* family (the genera of *Azospira* and *Dechloromonas*) (Gao et al. 2017b; Zhong et al. 2015). *Alphaproteobacteria*, including *Rhodobacter*, *Rhizobium* and *Hyphomicrobium*, etc. were also characterized as the important heterotrophic denitrifying bacteria. In addition, *Sulfurimonas*, as the most commonly reported autotrophic denitrifying bacteria (Zhong et al. 2015), were also detected in this investigation. In the VF, *Hydrogenophaga* and *Flavobacterium*, with sequences of 1,349 and 1,312 respectively, were detected as the dominant denitrifying genera. *Hydrogenophaga* and *Flavobacterium*, as the main heterotrophic denitrifying bacteria mainly accumulated in the area of VF1-1. This might be due to the relatively high COD concentration (abundant electron donors) and low DO concentration (anaerobic-anoxic environmental conditions) in the area of VF1-1 (Wu et al. 2016; Xu et al. 2016). In the HF, *Rhodobacter*, *Flavobacterium*, *Dechloromonas* and *Sulfurimonas*, with sequences of 949, 1,364, 1,359 and 1,233 respectively, were also detected as the dominant denitrifying genera. *Rhodobacter* and *Flavobacterium* were the predominant genera in HF1-1, while *Dechloromonas* and *Sulfurimonas* were the dominant genera in HF1-2. Heterotrophic and autotrophic denitrifying bacteria showed higher diversity and richness in the HF than in the VF.

Relative influences of water physicochemical properties on microbial community

Microbial community structure, especially nitrifying and denitrifying bacteria, could be easily influenced by wastewater variables such as COD, TN, pH, DO, etc. (Mietto et al. 2015). In this investigation, redundancy analysis was

Table 1 | Number of sequences belonging to the genera that might be involved in nitrogen removal

Processes	Genera	Sequence numbers			
		VF1-1	VF1-2	HF1-1	HF1-2
Nitrification (Zhong <i>et al.</i> 2015)	<i>Nitrosomonas</i>	5	6	3	17
	<i>Nitrospira</i>	53	8	427	63
Denitrification (Zhong <i>et al.</i> 2015; Gao <i>et al.</i> 2017b; Wang <i>et al.</i> 2017)	<i>Hyphomicrobium</i>	1	0	37	2
	<i>Rhizobium</i>	14	2	3	1
	<i>Rhodobacter</i>	93	35	949	101
	<i>Acidovorax</i>	225	62	27	88
	<i>Hydrogenophaga</i>	1,349	177	137	193
	<i>Azospira</i>	5	15	15	111
	<i>Dechloromonas</i>	380	142	25	1,359
	<i>Thauera</i>	1	1	0	0
	<i>Arcobacter</i>	0	2	0	105
	<i>Flavobacterium</i>	1,312	274	1,364	260
	<i>Sulfurimonas</i>	1	48	0	1,233
	<i>Thiobacillus</i>	0	1	0	2

used to reveal the relationship between the dominant bacteria in the four samples of the VF-HF system (VF1-1, VF1-2, HF1-1 and HF1-2) with environmental factors. Dominant bacteria involved in nitrogen removal at family (*Comamonadaceae*, *Xanthomonadaceae* and *Rhodocyclaceae*) and major function genera level (*Hydrogenophaga*, *Dechloromonas*, *Flavobacterium* and *Sulfurimonas*) were selected as target microorganisms. Environmental factors included the main wastewater indicators concerning nitrogen transformation (COD, $\text{NH}_4^+\text{-N}$ and TN) and the environmental physicochemical parameters (pH, DO and ORP) (Table S4, available online). Due to the concentrations of $\text{NO}_3^-\text{-N}$ being very low and the fact that the number of environmental factors should be less than the number of samples in redundancy analysis, we selected the $\text{NH}_4^+\text{-N}$ and TN for redundancy analysis.

The redundancy analyses (RDA) between water physicochemical properties and the top 15 microbial families from 16S rRNA MiSeq sequencing in the different functional areas within the VF-HF system is shown in Figure 4. As was discussed above, different functional microorganisms accumulated in the different areas of VF-HF system. Moreover, Figure 4(a) suggests that *Gemmatimonadaceae* showed a positive correlation with TN. *Desulfomicrobiaceae* and *Comamonadaceae* showed a positive correlation with COD, while *Lentimicrobiaceae* showed a positive correlation with $\text{NH}_4^+\text{-N}$. Figure 4(b) suggests that *Xanthomonadaceae* and *Saprospiraceae* showed a positive correlation with DO. *Xanthomonadaceae*, *Rhodobacteraceae*, *Erythrobacteraceae* and *Chitinophagaceae* showed a positive correlation with pH, while *Parcubacteria*,

Gracilibacteria, *env.OPS_17* and *Anaerolineaceae* showed a positive correlation with ORP. For the dominant denitrifying bacteria such as *Comamonadaceae*, *Xanthomonadaceae* and *Rhodocyclaceae*, Figure 4(a) reveals that *Comamonadaceae* was more positively related to COD and $\text{NH}_4^+\text{-N}$. This indicated that the effects of COD and $\text{NH}_4^+\text{-N}$ on nitrogen removal bacteria in the VF was higher than in the HF. Figure 4(b) reveals that *Xanthomonadaceae* was positively related to pH and DO, and *Rhodocyclaceae* was positively related to ORP. In addition, DO ($p = 0.042$) was most correlated to the community variances in the VF-HF system. The significance of these relationships was confirmed by the Monte Carlo permutation test. In conclusion, the effects of pH, DO and ORP on nitrogen removal bacteria in the HF were higher than in the VF.

Figure 5 further shows the interrelation between 14 genera (Table 1) involved in nitrogen removal with the physicochemical properties of the VF and the HF. Results suggested that high sequences of heterotrophic denitrifying bacteria were enriched in the VF, and high sequences of both heterotrophic and autotrophic denitrifying bacteria were enriched in the HF. In addition, higher nitrifying bacteria sequences were also observed to accumulate in the HF. The dominant genera of *Hydrogenophaga*, *Acidovorax*, *Thauera* and *Rhizobium* accumulated in the VF (Figure 5(a)) were more influenced by COD and $\text{NH}_4^+\text{-N}$ than other genera, and *Flavobacterium* was positively correlated with TN. These results demonstrated that the effects of COD and $\text{NH}_4^+\text{-N}$ on nitrogen removal bacteria in the VF were higher than in the HF. Figure 5(b) shows that pH and DO have the greater positive effect on *Flavobacterium*,

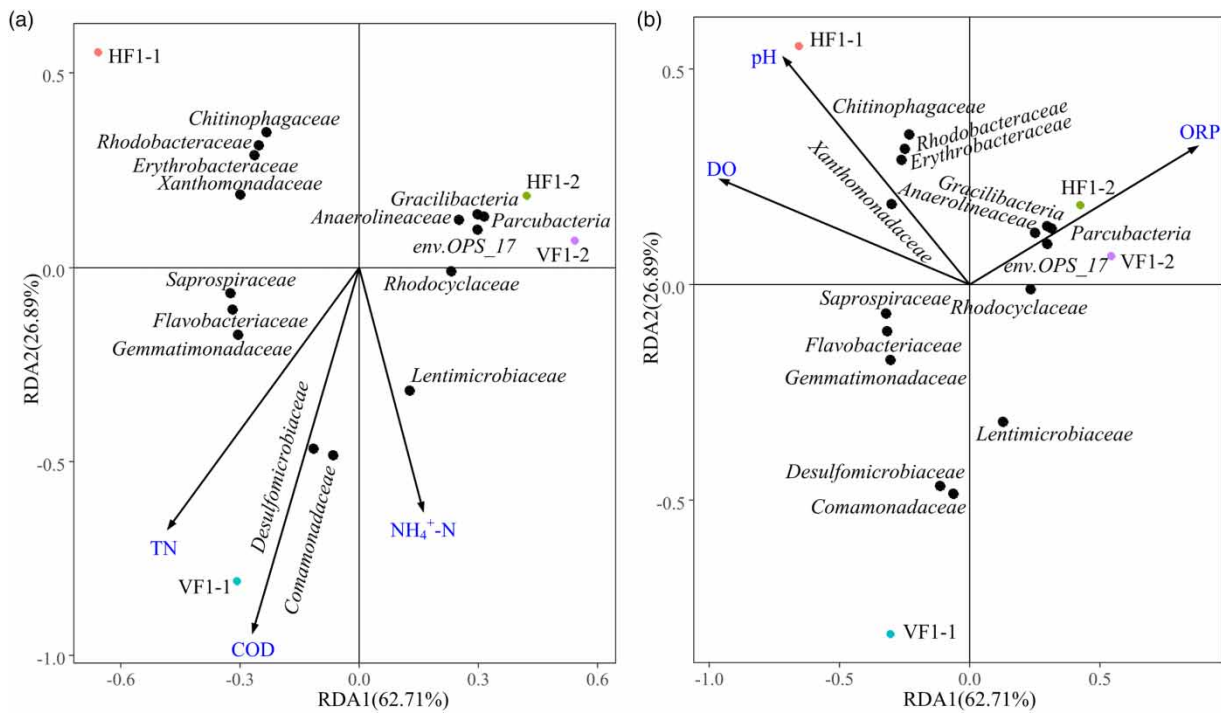


Figure 4 | Biplot of the RDA of the top 15 microbial families with water physicochemical values of four sampling areas (VF1-1, VF1-2, HF1-1 and HF1-2) in the VF-HF system: (a) COD, $\text{NH}_4^+\text{-N}$ and TN; (b) DO, pH and ORP.

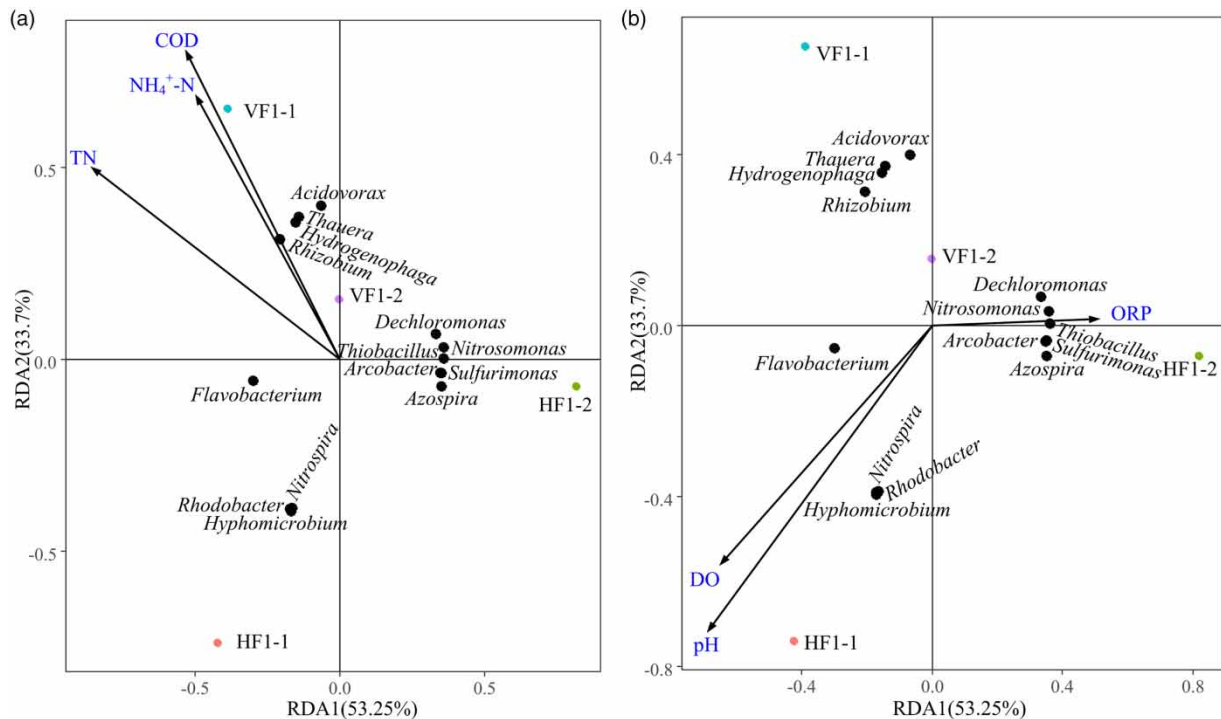


Figure 5 | Biplot of the RDA of the functional genera involved in nitrogen removal with water physicochemical values of four sampling areas (VF1-1, VF1-2, HF1-1 and HF1-2) in the VF-HF system: (a) COD, $\text{NH}_4^+\text{-N}$ and TN; (b) DO, pH and ORP.

Nitrospira, *Rhodobacter* and *Hyphomicrobium* than other genera, and ORP exerted an obvious influence on *Thiobacillus*, *Nitrosomonas*, *Arcobacter* and *Sulfurimonas*. The nitrifying bacteria were principally accumulated in the HF, indicating that the effects of pH, DO and ORP on nitrogen removal bacteria in the HF were higher than in the VF. In brief, the differences in COD, $\text{NH}_4^+\text{-N}$ and TN removal within the VF and HF were mainly due to the distribution of pollutants removal microorganisms in the system. Water physicochemical properties such as COD, $\text{NH}_4^+\text{-N}$, pH, DO, ORP, etc. also had a great influence upon the distribution of bacteria, especially nitrogen removal bacteria.

CONCLUSIONS

The VF-HF system exhibited high contaminant removal efficiency during treatment of the second effluent from the WWTP. Average removal efficiencies of 67.02% COD, 89.80% $\text{NH}_4^+\text{-N}$, 90.31% TN and 75.38% TP were achieved during the stable operation period. The VF showed much higher average loading rates of COD, TP, $\text{NH}_4^+\text{-N}$ and TN (7.96 g/m²/d, 0.076 g/m²/d, 0.31 g/m²/d and 0.99 g/m²/d) than in the HF (0.65 g/m²/d, 0.016 g/m²/d, 0.25 g/m²/d and 0.50 g/m²/d), during the stable operation period. The nitrification-denitrification process was the major removal pathway for nitrogen. Microbial community analysis demonstrated that heterotrophic denitrifying bacteria, such as *Hydrogenophaga* and *Flavobacterium*, were the dominant contributors to nitrogen removal in the VF. The heterotrophic denitrifying bacteria, such as *Rhodobacter*, *Flavobacterium* and *Dechloromonas*, as well as the autotrophic denitrifying bacteria *Sulfurimonas*, played the important roles for nitrogen removal in the HF. Redundancy analysis showed that COD and $\text{NH}_4^+\text{-N}$ were the important factors affecting the distribution of nitrogen removal bacteria in VF, while pH, DO and ORP were the key factors influencing the distribution of nitrogen removal bacteria in HF.

ACKNOWLEDGEMENTS

This work was supported by the National Water Pollution Control and Treatment Science and Technology Major Project of China (No. 2017ZX07203-003 and No. 2017ZX07204-002) and the Fundamental Research Funds for the Central Universities (No. JUSRP1703XNC).

REFERENCES

- Adrados, B., Sánchez, O., Arias, C. A., Becares, E., Garrido, L., Mas, J., Brix, H. & Morato, J. 2014 Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. *Water Research* **55**, 304–312.
- APHA 2005 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.
- Domingos, S. S., Dallas, S., Skillman, L., Felstead, S. & Ho, G. 2011 Nitrogen removal and ammonia-oxidising bacteria in a vertical flow constructed wetland treating inorganic wastewater. *Water Science & Technology* **64** (3), 587–599.
- Dong, H. Y., Qiang, Z. M., Li, T. G., Jin, H. & Chen, W. D. 2012 Effect of artificial aeration on the performance of vertical-flow constructed wetland treating heavily polluted river water. *Journal of Environmental Sciences* **24** (4), 596–601.
- Gao, L., Zhou, W. L., Huang, J. C., He, S. B., Yan, Y. J., Zhu, W. Y., Wu, S. Q. & Zhang, X. 2017a Nitrogen removal by the enhanced floating treatment wetlands from the secondary effluent. *Bioresource Technology* **234**, 243–252.
- Gao, Y., Xie, Y. W., Zhang, Q., Wang, A. L., Yu, Y. X. & Yang, L. Y. 2017b Intensified nitrate and phosphorus removal in an electrolysis-integrated horizontal subsurface-flow constructed wetland. *Water Research* **108**, 39–45.
- Jin, Z., Ji, F. Y., Xu, X., Xu, X. Y., Chen, Q. K. & Li, Q. 2014 Microbial and metabolic characterization of a denitrifying phosphorus-uptake/side stream phosphorus removal system for treating domestic sewage. *Biodegradation* **25**, 777–786.
- Li, H., Xu, X. Y., Li, P. J., Yin, W. & Verkhovzina, V. A. 2008 Research on ammonibacteria removing organic nitrogen in construction wetland. *Chinese Journal of Environmental Engineering* **2**, 1044–1047.
- Lu, S. Y., Wan, Z. F., Li, F. M. & Zhang, X. Q. 2016 Ammonia nitrogen adsorption and desorption characteristics of twenty-nine kinds of constructed wetland substrates. *Research of Environmental Sciences* **29** (08), 1187–1194.
- Mietto, A., Politeo, M., Bresschigliaro, S. & Borin, M. 2015 Temperature influence on nitrogen removal in a hybrid constructed wetland system in Northern Italy. *Ecological Engineering* **75**, 291–302.
- Ong, S. A., Uchiyama, K., Inadama, D. & Yamagiwa, K. 2009 Simultaneous removal of color, organic compounds and nutrients in azo dye-containing wastewater using up-flow constructed wetland. *Journal of Hazardous Materials* **165** (1–3), 696–703.
- Pramanik, B. K., Roddick, F. A., Fan, L., Jeong, S. & Vigneswaran, S. 2015 Assessment of biological activated carbon treatment to control membrane fouling in reverse osmosis of secondary effluent for reuse in irrigation. *Desalination* **364**, 90–95.
- Tao, W. D., Sauba, K., Fattah, K. P. & Smith, J. R. 2017 Designing constructed wetlands for reclamation of pretreated wastewater and stormwater. *Reviews in Environmental Science & Bio/Technology* **16**, 37–57.
- Vymazal, J. & Kröpfelová, L. 2011 A three-stage experimental constructed wetland for treatment of domestic sewage:

- first 2 years of operation. *Ecological Engineering* **37**, 90–98.
- Vymazal, J. & Kröpfelová, L. 2015 Multistage hybrid constructed wetland for enhanced removal of nitrogen. *Ecological Engineering* **84**, 202–208.
- Wang, P., Yu, Z. S., Qi, R. & Zhang, H. X. 2016 Detailed comparison of bacterial communities during seasonal sludge bulking in a municipal wastewater treatment plant. *Water Research* **105**, 157–166.
- Wang, J. F., Wang, Y. H., Bai, J. H., Liu, Z. W., Song, X. S., Yan, D. M., Asamimew, A., Zhao, Z. M. & Yan, D. H. 2017 High efficiency of inorganic nitrogen removal by integrating biofilm-electrode with constructed wetland: autotrophic denitrifying bacteria analysis. *Bioresource Technology* **227**, 7–14.
- Wu, Y. H., Han, R., Yang, X. N., Fang, X. H., Chen, X., Yang, D. & Zhang, R. D. 2016 Correlating microbial community with physicochemical indices and structures of a full-scale integrated constructed wetland system. *Applied Microbiology and Biotechnology* **100**, 6917–6926.
- Xia, X. H., Liu, T., Yang, Z. F., Zhang, X. Q. & Yu, Z. B. 2013 Dissolved organic nitrogen transformation in river water: effects of suspended sediment and organic nitrogen concentration. *Journal of Hydrology* **484**, 96–104.
- Xu, M., Liu, W. J., Li, C., Xiao, C., Ding, L. L., Xu, K., Geng, J. J. & Ren, H. Q. 2016 Evaluation of the treatment performance and microbial communities of a combined constructed wetland used to treat industrial park wastewater. *Environmental Science and Pollution Research* **23**, 10990–11001.
- Yang, C., Li, L., Shi, J., Long, C. & Li, A. 2015 Advanced treatment of textile dyeing secondary effluent using magnetic anion exchange resin and its effect on organic fouling in subsequent RO membrane. *Journal of Hazardous Materials* **84**, 50–57.
- Zhong, F., Wu, J., Dai, Y. R., Yang, L. H., Zhang, Z. H., Cheng, S. P. & Zhang, Q. 2015 Bacterial community analysis by PCT-DGGE and 454-pyrosequencing of horizontal subsurface flow constructed wetlands with front aeration. *Applied Microbiology and Biotechnology* **99**, 1499–1512.
- Zhou, X. H., Wang, G. X. & Yang, F. 2011 Characteristics of growth, nutrient uptake, purification effect of *Ipomoea aquatica*, *Lolium multiflorum*, and *Sorghum sudanense* grown under different nitrogen levels. *Desalination* **273**, 366–374.
- Zhou, X., Wang, X. Z., Zhang, H. & Wu, H. M. 2017 Enhanced nitrogen removal of low C/N domestic wastewater using a biochar-amended aerated vertical flow constructed wetland. *Bioresource Technology* **241**, 269–275.

First received 21 January 2018; accepted in revised form 17 May 2018. Available online 1 June 2018