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RESEARCH ARTICLE

Enhanced nitrogen removal in filled-and-drained vertical flow constructed wetlands: microbial responses to aeration mode and carbon source

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Abstract

For the purpose of enhancing the removal rate of nitrogen (N) and organic matters, intermittent aeration and carbon source were used in filled-and-drained vertical flow constructed wetlands (VFCWs). The results showed that the best removal of COD (74.16%), NH_4^+-N (93.56%), TN (86.88%), and NO_3^--N (79.65%) was achieved in VFCW1 (aerated with carbon source system). Illumina MiSeq300 high-throughput sequencing showed that carbon source aerated system increases the diversity and richness of the microbial community. The copy numbers of nitrification functional genes (*nxrA*, *amoA*), denitrification functional genes (*nirS*, *nirK*, *nosZ*), and anammox functional gene (anammox 16S rRNA) displayed various changes when applied different aeration modes and additional carbon source to each system. An increase of the DO concentration and carbon source facilitated the absolute abundance of microbial nitrification and denitrification functional genes, respectively. All in all, these results demonstrate that carbon source combined with intermittent aeration is valid to improve the pollutant treatment performance in these systems.

Keywords Constructed wetlands · Nitrogen removal · Carbon source · Intermittent aeration · High-throughput sequencing · Functional genes

Introduction

High N and organic matters released into water bodies lead to deterioration of water quality via eutrophication (Fan et al. 2016). Compared with the traditional sewage treatment technology, the constructed wetlands (CWs) have the characteristics of stable effluent quality, low energy consumption, simple operation, and environmentally friendly performance (Hou et al. 2018). Thus, it is widely used throughout the world. Due to the high pollutant removal efficiency, good oxygen transfer performance for nitrification, and a small footprint, VFCWs are often applied to purify and treat various wastewaters (Pelissari et al. 2017). The CWs utilize a substrate–plant–microbial integration system under the combination of physical, chemical, and biological effects (Liu et al. 2018) to achieve efficient purification of sewage by precipitation and volatilization, microbial degradation, plant absorption, substrate adsorption, and filtration (Wang et al. 2016a, b; Jia et al. 2018). CWs perform beneficial treatment effect on organics and suspended solid (SS), but removal rate of N in wastewater shows enormous fluctuations (Wang et al. 2014). Consequently, enhancing N removal rate has being an issue in CW continuous wastewater treatment.

Microbial transformation (nitrification and denitrification) is one of the main N removal mechanisms in CWs (Mesquita et al. 2017). Nitrification is the process of oxidizing ammonia nitrogen (NH_4^+-N) to nitrate nitrogen (NO_3^--N) by nitrifying bacteria under aerobic conditions (Zhou et al. 2017).

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Denitrification refers to the process in which nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) are converted into gaseous nitrogen (N_2) by the transformation of denitrifying bacteria (Tan et al. 2017). The minimal utilization rate of dissolved oxygen (DO) is one of the main factors which lead to poor N removal in wetlands (Li et al. 2014). Adequate DO supply is the key to ensure the nitrification process by facilitating the multiplication of the nitrobacteria (Jia et al. 2018). The intermittent aeration operation mode of CWs can improve the DO level in the wetlands and create an alternate aerobic and anoxic environment to promote nitrification and denitrification, respectively, thereby achieving simultaneous efficient removal of NH_4^+-N and TN (Wu et al. 2015; Zhou et al. 2017). In addition, carbon source is one of the important inhibition factors which affect the denitrification in CWs during microbial heterotrophic metabolism (Saeed et al. 2018). When the COD/N ratio in wastewater is low, an exogenous carbon source is required to provide denitrifying electron donors (Zhou et al. 2017; Pan et al. 2017). Therefore, studying the combination of intermittent aeration and carbon source is essential for the enhancement of the N removal from wastewater in CWs.

The conversion, circulation, and removal of N and organic pollutants in CWs are closely linked to the microbial species, quantity, and spatial and temporal distribution in the systems (Zhu et al. 2014). Therefore, microorganism transformation is deemed to be the dominant route of N removal (Li et al. 2017). Moreover, the degradation of pollutants in water is mainly implemented by microorganisms, which act as the primary decomposer in the system (Faulwetter et al. 2009). It has a close relationship between the microbial biomass and purification functions in CWs. The increase of microorganism biomass leads to an improvement in the removal rate of pollutants (Hu et al. 2016). Previous study found that the amount of microorganisms in the sewage, especially the number of nitrifying and denitrifying bacteria, is positively correlated with the efficiency of N removal (Zhu et al. 2014). In addition, Wang et al. (2015) reported that the functional genes of *nxrA* (nitrite oxidoreductase), *amoA* (ammonia monooxygenase), anammox bacterial 16S rRNA (anammox bacteria), *nirS* (cd1-containing nitrite reductase), *nirK* (copper-containing nitrite reductase), and *nosZ* (nitrous oxide reductase) participated in microbial nitrification and denitrification processes. Up to now, there are few reports on the microbial responses during N removal through the operation of intermittent aeration combined with exogenous carbon addition in influent. Therefore, in order to reveal the mechanism and process of N removal from wetlands, it is highly necessary for us to determine the copy number of N transformation functional genes in the system by real-time quantitative PCR technology. Hence, a comprehensive understanding of the relationships between N transformation functional genes which indicate the varieties of microbial functions and N removal efficiency can help optimize the treatment performance of pollutants in VFCWs.

This study addressed the effects of intermittent aeration combined with exogenous carbon source on N removal efficiency and microbial responses in VFCWs. The main purpose of this experiment were to: (1) explore the optimal removal rate of N and organic matters; (2) investigate the variety of microorganism community as influenced by operation conditions; and (3) quantify the affiliation between microbial responses and removal of corresponding pollutants.

Materials and methods

Description and operation of VFCWs

In this experiment, four filled-and-drained VFCWs of the same size were designed and placed outdoor in the University of Jinan, which were recorded as VFCW1 (aerated with carbon source addition), VFCW2 (aerated with non-carbon source addition), VFCW3 (non-aerated with carbon source addition), and VFCW4 (non-aerated with non-carbon source addition). All experimental systems were prepared by Plexiglass (50 cm in length, 40 cm in width, and 60 cm in height) with a working volume of 35 L. The diagrammatic sketch of the VFCW systems was displayed in Fig. 1. The systems were packed according to our previous study (Lai et al. 2020). The substrates were divided into four layers from top to bottom with 10-cm-thick soils, 10-cm-thick quartz sands (1–2 mm in diameter), 15-cm-thick zeolites (8–10 mm in diameter), and 15-cm-thick lava rock (8–10 mm in diameter). The arrangement of the substrates from small to large in size increases the utilization of aeration. *Canna indica* L. was planted in each system at a density of 12 plants. There are two pre-buried PVC pipes which were located in VFCW system center. One (10 cm in diameter) was applied for pH and DO value determination in situ, and the other (3 cm in diameter) was for artificial aeration, which was connected to an air pump and perforated on the pipe body at intervals of 10 cm.

This experiment began in June 2018 and lasted a total of 150 days. Before the artificial contaminated influent was imported, the systems have irrigated with secondary wastewater received from Everbright Water Co., Ltd. (Jinan, China) for one month for the proliferation of microorganisms. So, as to investigate the N removal efficiency of VFCWs, different aeration modes and carbon source addition were designed. As an external carbon source, glucose was added to the systems. Synthetic wastewater was made up of tap water (Jinan) and mainly contained the following components (per liter): 0.0049 g KNO_3, 0.089 g NH_4Cl, and 0.19 g glucose, leading to concentrations of 3.6 mg L^{-1} NO_3^-, 30.8 mg L^{-1} NH_4^+, and 196 mg L^{-1} COD, and the ratio of COD/N achieved in 5.87, which were applied to minimize inconstancy of the influent

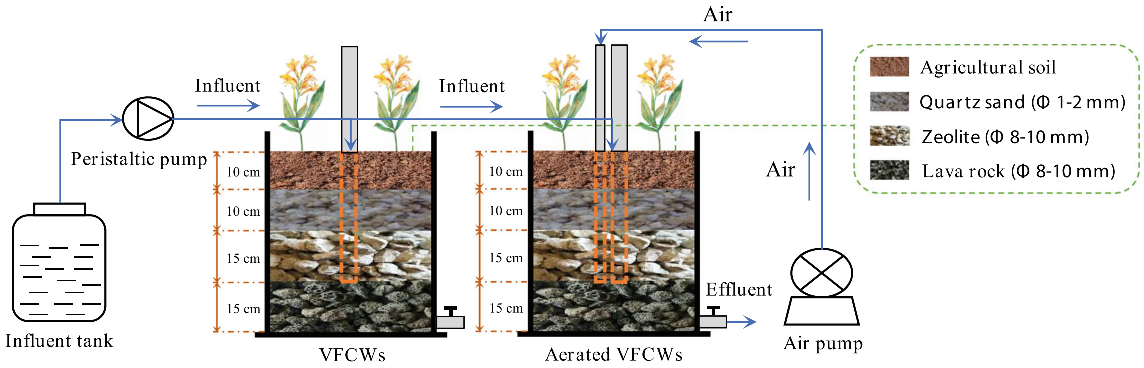

Fig. 1 The diagrammatic sketch of the VFCW systems

concentration. The detailed compositions in the synthetic wastewater have been demonstrated by Lai et al. (2020). The VFCW system which required aeration was intermittently aerated for 4 h at an air-flow rate of 1.0 ± 0.2 L $\min^{-1}$ every day. After every hydraulic retention time (HRT, 72 h) of the cycle, each VFCW was drained and immediately refilled with the wastewater, which formed a filled-and-drained batch mode. In the current operating mode, hydraulic loading rate (HLR) of the tested VFCW system during the whole study was 0.058 m^3 (m^{-2} d^{-1}).

Water sample collecting and testing

Influent and effluent samples from each VFCW were collected every 72 h, hereafter, transferred immediately to lab for analysis of COD, NH_4^+-N, TN, NO_3^--N, and NO_2^--N in the light of standard methods (APHA 2005). The DO and pH value were measured in situ with a digital DO meter (CyberScan DO110, USA) and pH glass meter (DENVER UB-7, USA), respectively.

Microbial communities and qPCR analysis

Sampling of microorganisms

The substrates samples were collected at the center and in four corners, positions of the VFCW systems, and homogenized to obtain a composite sample after 150 days of system operation. The sample was stored in a sterile plastic bag placed in an ice incubator and brought back to the laboratory for further processing. Then, the substrate samples were washed by vortexing. Lastly, it was centrifuged at 12,000*g* for 2 min to collect the sediment in sterile tubes (Du et al. 2017). Sediment of the substrate samples was stored at − 20 °C for further analysis and testing.

Illumina MiSeq high-throughput sequencing

E.Z.N.A. soil DNA kit (Omega, USA) was utilized to extract DNA from diverse microbial samples in accordance with the manufacturer's instructions. The extracted DNA was detected by way of 2% agarose gel electrophoresis and preserved at − 20 °C until use. Microbial sample analysis by Illumina MiSeq high-throughput sequencing was provided by LC-Bio Technology Co., Ltd. (Hangzhou, China). Operational taxonomic units (OTUs) were clustered via Vsearch (version 2.3.4) at 97% sequences similarity. To understand microbial diversity and community richness, the indices of Simpson, Observed species, Shannon, and Chao 1 were calculated by Qiime (version 1.8.0). Taxonomic datum was allocated at the phylum level through ribosomal database project (RDP).

Quantitative polymerase chain reaction

DNeasy PowerSoil Kit (MoBio, USA) was utilized to extract the microbial genomic DNA. Quantitative testing of related transformation functional genes (*nxrA*, *amoA*, *nosZ*, *nirS*, *nirK*, and anammox 16S rRNA) was conducted on a real-time quantitative PCR detection system (Bio-Rad, USA). Synthetic primers were presented in Table S1 and diluted according to the manufacturer's (Nanjing Jinsirui Biotechnology Co., Ltd., Nanjing, China) instructions. The 20 μL reaction mixture had the following composition: 10 μL Maxima SYBR Green *q*PCR Master Mix (2×) (TaKaRa), 1 μL template DNA (sample DNA or plasmid DNA for standard curves), 0.4 μL in forward and reverse primers, and sterile water. Quantitative analysis was conducted using a procedure of three-step thermal cycle, followed by melting curve analysis. It is useful to note that all assays were performed in triplicate to reduce the variables.

Statistical analyses

Software SPSS 17.0 (SPSS Inc., Chicago, USA) was carried out for all statistical analyses performed by one-way analysis of variance (ANOVA). The statistically significant differences among treatment performances in three VFCW systems were considered at $P < 0.05$. Redundancy analysis (RDA) (Canoco 5.0) was used to illustrate the relationship between microorganisms and N removal.

Results and discussion

Overall removal performances of N and organic matters

N removal

During the experimental period, the average effluent concentration and removal efficiency of NH_4^+-N, NO_3^--N, and TN were shown in Fig. 2 and Table 1, respectively. With varied aeration modes and exogenous carbon source, VFCW systems presented different removal efficiency in N removal. Overall, there are significant differences among the N removal rate in VFCWs. As shown in Fig. 2 and Table 1, NH_4^+-N removal efficiency could achieve above 84% under all treatments, and the average effluent NH_4^+-N concentration fall below 5 mg L^{-1}, which was within the scope of Chinese wastewater discharge standard (GB18918-2002). The removal of NH_4^+-N mainly depends on microbial nitrification, which transforms NH_4^+-N to NO_3^--N under sufficient oxygen conditions (Li et al. 2014). NH_4^+-N removal rate of the aerated VFCW1 (93.56%) and VFCW2 (95.38%) was higher than those of the non-aerated VFCW3 (84.45%) and VFCW4 (85.37%) under unanimous exogenous carbon source ($P < 0.05$). Research of Yang et al. (2017) found that NH_4^+-N removal rate was higher in aerated CWs, which was similar to our findings. In the present study, the removal of NH_4^+-N decreased with the addition of a carbon source under the identical aeration mode. Owing to the addition of the carbon source, increasing organic matters consume part of the oxygen and the insufficient supply of oxygen prevents the NH_4^+-N from being completely converted into NO_3^--N (Zhou et al. 2019). Therefore, the reduction of NH_4^+-N removal may be due to hypoxia inhibition of nitrification (Zhu et al. 2014). The current study demonstrated that the combination of intermittent aeration and exogenous carbon source improved NH_4^+-N removal rate, which was more effective than the previous result (88%) achieved by Ding et al. (2014). Furthermore, the NH_4^+-N removal efficiency obtained by Zhao et al. (2016) was 84%, indicating the operation and setup in current VFCWs are conducive to the removal of NH_4^+-N.

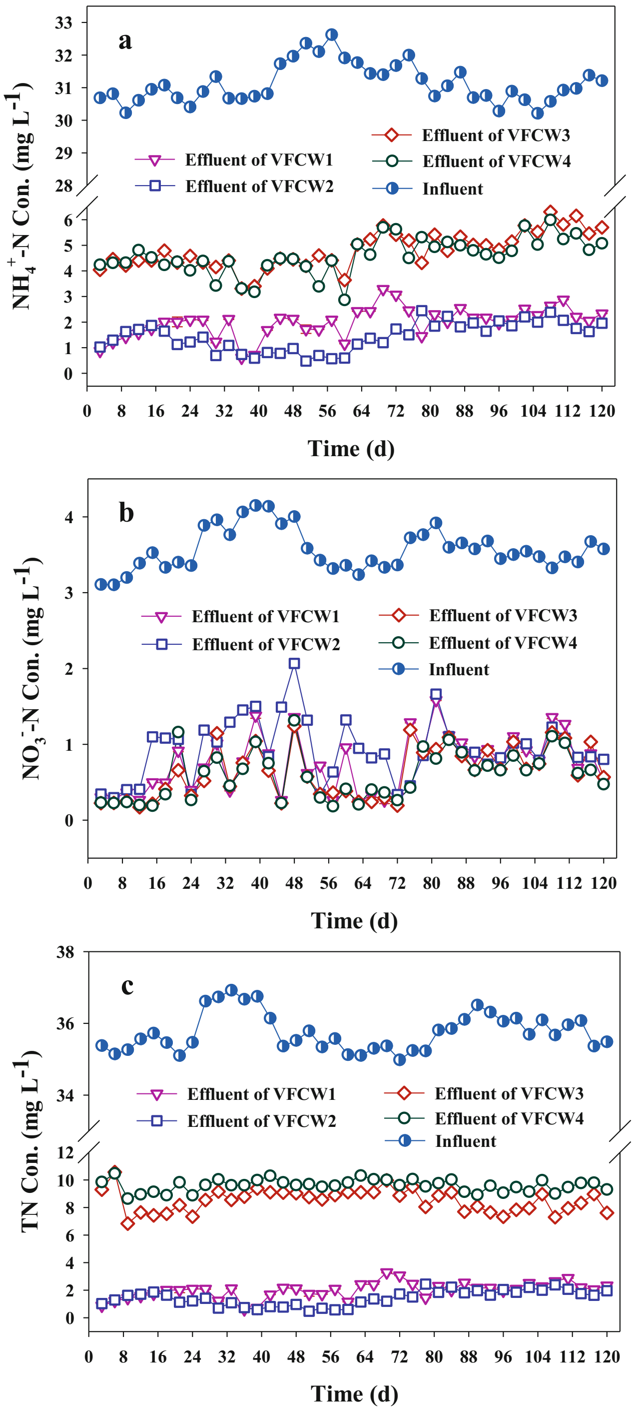

Fig. 2 Average effluent concentration and removal efficiency of COD in VFCWs. VFCW1, aerated with carbon source CW; VFCW2, aerated with non-carbon source CW; VFCW3, non-aerated with carbon source CW; VFCW4, non-aerated with non-carbon source CW

As seen from Fig. 2b, a lower average effluent NO_3^--N concentration was achieved in VFCW1 (0.75 mg L^{-1}) and VFCW3 (0.63 mg L^{-1}), suggesting the higher removal rate of NO_3^--N is mainly attributed to good denitrification in VFCW system with carbon source addition. Previous studies have observed that insufficient carbon sources or excessive oxygen inhibited denitrification (Wu et al. 2016), which supports our results. NO_3^--N removal mainly depends on microbial heterotrophic denitrification, which was conducted under anaerobic conditions (Pan et al. 2017). In this study, it revealed that the combined operation of intermittent aeration and carbon source addition provides oxygen condition and available carbon supply for aerobic nitrification and anaerobic denitrification of microorganisms, respectively. Thus, the highest NO_3^--N removal rate was obtained in VFCW3 (82.91%) among all systems. In addition, the removal rate of NO_3^--N under these running VFCWs were greater than those in non-aerated VFCWs packed with fly-ash brick (65%) (Liu et al. 2019) and the two-stage deep sequencing CW (NO_3^--N

Table 1 Respective effluent concentrations and removal efficiency in four VFCWs (mean ± SD, $n = 40$)

Parameters	Concentrations (mg L^{-1})						Removal efficiency (%)			
	COD	NH_4^+-N	NO_3^--N	TN	NO_2^--N	DO	COD	NH_4^+-N	NO_3^--N	TN
VFCW1	54.73 ± 5.77	1.99 ± 0.59	0.75 ± 0.38	4.74 ± 0.49	0.50 ± 0.25	5.75 ± 1.60	74.16 ± 2.82	93.56 ± 1.88	79.65 ± 9.73	86.88 ± 1.25
VFCW2	39.07 ± 6.00	1.44 ± 0.55	0.94 ± 0.40	5.48 ± 0.81	0.53 ± 0.29	5.58 ± 1.53	62.51 ± 5.70	95.38 ± 1.80	73.55 ± 10.22	84.51 ± 2.23
VFCW3	89.35 ± 3.73	4.80 ± 0.71	0.63 ± 0.34	8.50 ± 0.82	0.55 ± 0.31	3.54 ± 0.66	57.82 ± 1.77	84.45 ± 2.34	82.91 ± 8.73	76.45 ± 2.44
VFCW4	48.35 ± 3.99	4.58 ± 0.70	0.85 ± 0.23	9.59 ± 0.44	0.60 ± 0.34	3.20 ± 0.60	53.58 ± 4.07	85.37 ± 2.30	75.90 ± 6.05	72.89 ± 1.20

VFCW1, aerated with carbon source CW; VFCW2, aerated with non-carbon source CW; VFCW3, non-aerated with carbon source CW; VFCW4, non-aerated with non-carbon source CW

concentration accumulation) (He et al. 2018). This finding manifested that the operating conditions of these systems facilitate the growth of heterotrophic denitrifying bacteria. The concentration of NO_2^--N was all less than 0.60 mg L^{-1} during the whole operation period, indicating the nitrification and denitrification processes occurred thoroughly, although no statistically significant differences were discovered among four VFCW systems ($P > 0.05$).

In the current study, the average effluent concentration of TN was 7.08 mg L^{-1} and removal efficiency ranged from 72.9 to 86.9% (Fig. 2c and Table 1). Aeration modes significantly varied the TN removal efficiency ($P < 0.05$). In VFCW3 and VFCW4, low oxygen supply inhibited microbial nitrification in the system and led to insufficient NO_3^--N for denitrification, resulting in a low TN removal rate (10–11% lower than that with intermittent aeration). Yang et al. (2017) reviewed the previous results and indicated that the average removal rate of TN in the systems of aeration was greater than the non-aeration systems (97.03 to 90.41%). Similar research results also appeared in the study by Zhou et al. (2017), which concluded that hypoxia inhibited the removal of TN. Compared with VFCW2, removal rate of TN in VFCW1 significantly increased due to the addition of a carbon source ($P < 0.05$). A significantly higher TN removal rate was also found in VFCW3 than VFCW4 ($P < 0.05$) (Table 1). The addition of a carbon source promoted the heterotrophic denitrification process, thereby increasing the removal efficiency of TN (Zhi and Ji 2014). Recent findings from Wu et al. (2018) achieved analogous conclusions, suggesting the removal efficiency of TN reached a higher value when the cattail litter was used as an exogenous carbon source. In addition, the removal efficiency of TN under these running VFCWs was greater than those in biochar-intermittent aerated VFCW (53%) (Zhou et al. 2017), the bioaugmentation VFCW (74%) (Zhao et al. 2016), and polyvinyl alcohol immobilized nitrifier CW (43%) (Wang et al. 2016a, b). The highest TN removal efficiency (86.88%) was obtained in VFCW1 (intermittently aerated with carbon source) (Table 1), which verified that carbon source supplement in combination with artificial intermittent aeration enhanced the TN removal efficiency. In the existing studies, there is always a huge difference in the highest TN removal efficiency, which may be caused by various operating conditions, for instance the types of the CWs, climate environment, cultivated plants, and substrate filling materials (Gargallo et al. 2017). Therefore, studies on a wider range of designed continuous water are needed to assess the impact of operational conditions, especially on the effects of aeration combined with carbon source addition on microbial functions, which are associated with the N removal.

COD removal

As showed in Table 1, the DO concentration in VFCW1 (5.75 mg L^{-1}) and VFCW2 (5.58 mg L^{-1}) increased significantly due to the intermittent aeration. In contrast, low DO was detected in VFCW3 (3.54 mg L^{-1}) and VFCW4 (3.20 mg L^{-1}), which resulted in an anaerobic environment with a lack of oxygen supply. Zhou et al. (2017) concluded that DO is a crucial factor in the degradation of organic matters. Figure 3 showed the average COD removal rate and concentration in effluent in four experimental VFCWs. Among the four VFCWs, the COD removal efficiency in intermittent aeration systems (VFCW1 and VFCW2) was much higher than non-intermittent aeration systems (VFCW3 and VFCW4). An adequate supply of oxygen provided by the intermittent aeration led to higher COD removal efficiency. The results resemble with the study conducted by Yang et al. (2017), who investigated that the average concentration of effluent COD was higher in CWs with non-aeration (72.76 mg L^{-1}). In this study, COD removal efficiency was 74.16% in VFCW1, which was greater than that in VFCW2 (62.51%). Similar trends were also observed in VFCW3 (57.82%) and VFCW4 (53.58%). Previous studies have investigated that the removal rate of COD increased when the amount of exogenous carbon source increased in the influent in the VFCW systems (Jia et al. 2018). Therefore, we have confirmed that the addition of a carbon source enhances the removal of COD in the wastewater. Furthermore, we also found that the removal rate of COD in bioaugmentation VFCW was 65% (Zhao et al. 2016), which was lower than that of intermittently aerated external carbon addition VFCW (74%) in this study, indicating the operation conditions in this system are conducive to the removal of organic matters.

Microorganism communities

Microbial community diversity and abundance

Illumina Miseq high-throughput sequencing was conducted to analyze the impact of aeration modes and carbon source addition on microbial community structure on substrates of biofilm in CWs. The microbial richness (Chao1 index) and diversity (Shannon index and Simpson index) in different systems were shown in Table S2 (Supplementary Information). The observed species represent the number of OTUs in the samples. The observed species and Chao1 index were greater in aeration system (VFCW1 and VFCW2) than corresponding values of non-aeration systems (VFCW3 and VFCW4), which indicated that aeration increased the microbial richness. Moreover, higher Shannon and Simpson indices were also obtained in aeration system, indicating the microbial diversity was facilitated by aeration. Among the two aerated systems, the values of observed species (3569.33), Chao1 index (5435.32), Shannon index (9.62), and Simpson index (0.99) were higher in VFCW1 than the corresponding values of VFCW2 (3438, 5250.37, 9.45, and 0.99), respectively. Similar trends were shown in VFCW3 and VFCW4, which pointed out that carbon sources increase the richness and diversity of microorganisms. In summary, the indices of Shannon, Chao1, and Simpson in these four VFCW systems were significantly higher than those of ofloxacin CWs (Tong et al. 2019) and free water surface CWs (Zheng et al. 2018). It proved that a combination of intermittent aeration and exogenous carbon source was beneficial to microbial growth and the increase of bacterial diversity in the VFCW systems.

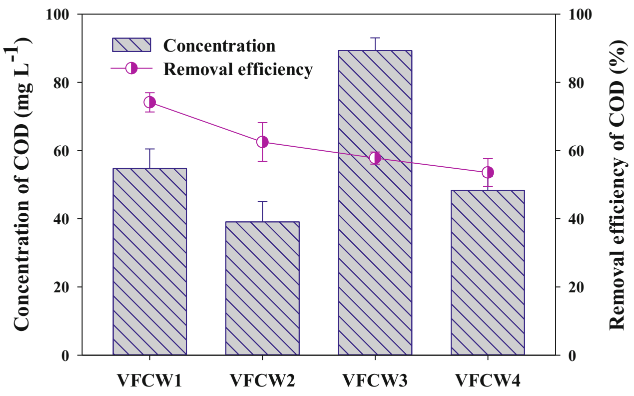

Fig. 3 Nitrogen concentrations of influent and effluent ((**a**) NH_4^+-N, (**b**) NO_3^--N, and (**c**) TN) in four VFCWs throughout the whole experimental period. VFCW1, aerated with carbon source CW; VFCW2, aerated with non-carbon source CW; VFCW3, non-aerated with carbon source CW; VFCW4, non-aerated with non-carbon source CW

Microbial community composition

The identified top 10 bacteria phyla with relative abundance over 1% were illustrated in Fig. 4 to evaluate the impacts of aeration modes and carbon source addition on bacterial community composition. There were three major phyla with different relative abundance that appeared in all systems, in which *Proteobacteria* was the dominant phylum. The *Proteobacteria* in each system accounted for 36.98, 46.03, 38.17, and 35.00%, respectively. It has been reported that *Proteobacteria* played an important role in the transformation and cycling of carbon, N, and sulfur (Ansola et al. 2014; Wang et al. 2016a, b). *Actinobacteria* accounted for 20.58% in VFCW1, 12.04% in VFCW2, 16.71% in VFCW3, and 15.93% in VFCW4, followed by *Acidobacteria* which accounted for 10.90, 10.47, 11.10, and 11.01% of the above-mentioned four systems, respectively. Previous investigation indicated that *Bacteroidetes* and *Actinobacteria* could produce antibiotics to suppress plant pathogens and promote denitrification (Zhang et al. 2017). Figure 4 showed that the relative abundance of *Actinobacteria* in carbon source addition systems (VFCW1 and VFCW3) was significantly greater than systems (VFCW2 and VFCW4) with non-carbon source, which indicated that carbon source addition facilitated the growth of heterotrophic denitrifying bacteria. Previous study reported that *Acidobacteria* had the ability to degrade phosphorus and organics (Wang et al. 2016a, b). Furthermore, it

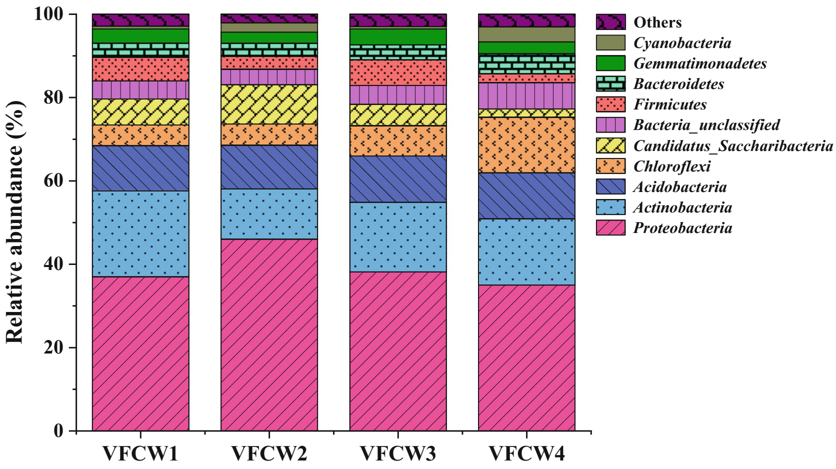

Fig. 4 The relative abundance of the bacterial communities at the main phylum level in VFCWs. VFCW1, aerated with carbon source CW; VFCW2, aerated with non-carbon source CW; VFCW3, non-aerated with carbon source CW; VFCW4, non-aerated with non-carbon source CW

should be noted that in the four systems, *Firmicutes* dominated higher abundance of 5.68, 3.05, 6.14, and 2.11%, respectively. Chen et al. (2017) disclosed that *Firmicutes* was a strictly heterotrophic denitrifying bacterium, which led to better NO_3^{-}-N removal efficiency in carbon source addition systems (VFCW1 and VFCW3).

N transformation functional genes

The number of functional genes provides information on the number of related microorganisms (Zhang et al. 2018). Throughout the current experiment, the absolute abundance of anammox bacterial (anammox bacterial 16S rRNA), nitrifying bacteria (*amoA*, *nxrA*), and denitrifying bacteria (*nosZ*, *nirK*, and *nirS*) were quantified to analyze microorganisms on the surface of the substrates in each VFCW.

Two genes of *nxrA* and *amoA* were used to evaluate the microbial abundance which participate in the nitrification process (Dionisi et al. 2002; Hu et al. 2016). *AmoA* gene codes ammonia monooxygenase, representing the transformation procedure from NH_4^{+}-N to NO_2^{-}-N (Johnston et al. 2019). Nitrite oxidase (*nxrA*) gene indicates the process of nitrite oxidation (NO_2^{-}-N converted to NO_3^{-}-N) (Attard et al. 2010). The absolute abundance of *amoA* gene was higher in aerated CW systems of VFCW1 and VFCW2 (2.64×10^5 and 3.98×10^5 copies g^{-1}), than non-aerated CW systems of VFCW3 and VFCW4 (1.20×10^5 and 1.63×10^5 copies g^{-1}) ($P < 0.05$) (Fig. 5a and Table S3). In the study of Yang et al. (2017), the copy number of *amoA* gene in the limited aeration CW (7.53×10^4 copies g^{-1}) was greater than that of the non-aerated CW (1.25×10^4 copies g^{-1}), indicating that the aeration enhanced microbial activity related to nitrification. The absolute abundance of *nxrA* displayed similar tendencies to those described by *amoA*. In aerated systems, the copy numbers of *nxrA* were 5.78×10^4 and 3.00×10^5 copies g^{-1}, which were greater than the values in non-aerated VFCW3 (2.05×10^4 copies g^{-1}) and VFCW4 (3.27×10^4 copies g^{-1}). Aeration promoted the activity of aerobic microorganisms and increased the abundance of aerobic-nitrifying bacteria, which is consistent with the increase of *amoA* and *nxrA* gene in the aeration system. The result was congruent with the previous research, which found that the copy number of *amoA* and *nxrA* increased with oxygen supply (Pan et al. 2017). In this study, it was found that the addition of a carbon source (VFCW1 and VFCW3) reduced the absolute abundance of functional genes *amoA* and *nxrA* (Table S3). The possible reason was that microbial nitrification was inhibited due to excess oxygen consumption (Chen et al. 2019).

Anammox 16S rRNA marks the anammox process which transforms NH_4^{+}-N and NO_2^{-}-N to N_2 by anammox bacteria under anaerobic conditions (Tsushima et al. 2007). Figure 5b showed the highest copy number (2.00×10^7 copies g^{-1}) obtained in VFCW4 (non-aerated with non-carbon source). The absolute abundance of anammox 16S rRNA in non-aerated systems (VFCW3 and VFCW4) was nearly 1 time greater than those in aerated systems (VFCW1 and VFCW3), suggesting anaerobic conditions are more conducive to the anammox process. Li et al. (2019) found that the contribution of anaerobic anammox 16S rRNA with the removal of NH_4^{+}-N increased due to the low concentration of DO in the systems. The copy number of anammox 16S rRNA declined as the addition of a carbon source, but the results did not show significant differences between treatments ($P > 0.05$). Therefore, we concluded that anaerobic anammox bacteria was barely affected by the carbon source addition under aerated or non-aerated operational conditions in this study. Our findings are consistent with the investigation conducted by Hu et al. (2016), who reported that anammox generated mainly in anaerobic area of the VFCWs.

Figure 5c showed the functional genes involved in microbial denitrification (*nosZ*, *nirK*, and *nirS*). *NirK* and *nirS* genes implement the nitrite reduction, which convert NO_2^{-}-N to N_2O and NO (Throbäck et al. 2004; Kandeler et al. 2006). Nitrous oxide reductase (*nosZ*) gene, an indicator of complete

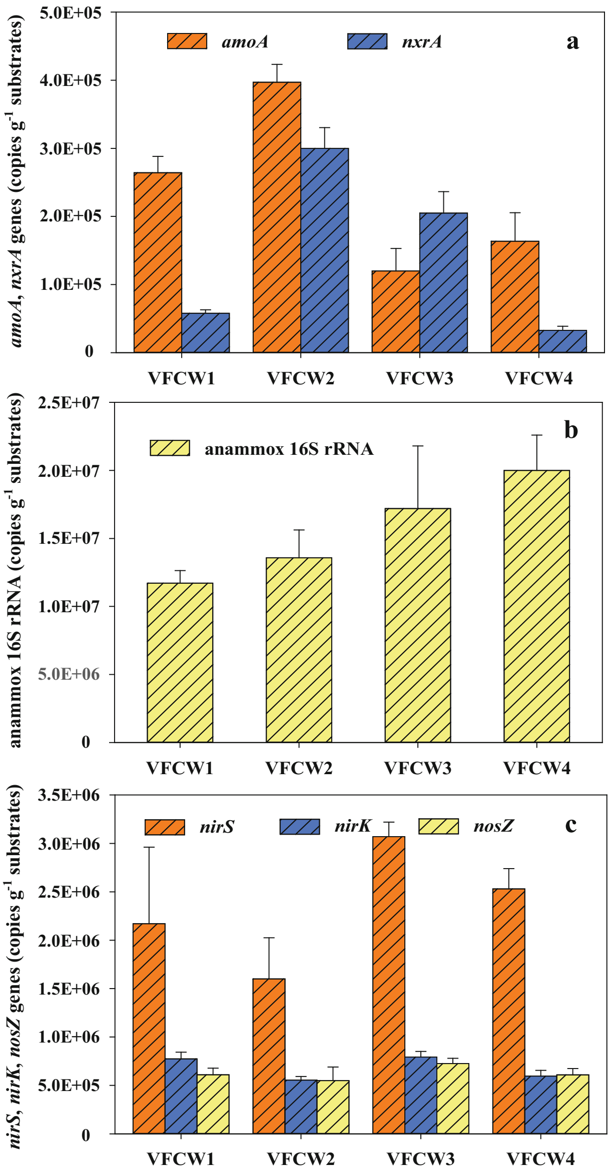

Fig. 5 The absolute abundance of related functional genes in each VFCW system: (**a**) nitrification bacteria (*amoA* and *nxrA*); (**b**) anammox 16S rRNA; (**c**) denitrification bacteria (*nirS*, *nirK*, and *nosZ*). VFCW1, aerated with carbon source CW; VFCW2, aerated with non-carbon source CW; VFCW3, non-aerated with carbon source CW; VFCW4, non-aerated with non-carbon source CW

heterotrophic denitrification, transforms N_2O and NO to N_2 (Henry et al. 2006). This present research displayed that the copy numbers of *nosZ*, *nirK*, and *nirS* were 6.10×10^5, 7.74×10^5, and 2.17×10^6 copies g^{-1} in VFCW1, 5.50×10^5, 5.55×10^5, and 1.60×10^6 copies g^{-1} in VFCW2, 7.26×10^5, 7.93×10^5, and 3.07×10^6 copies g^{-1} in VFCW3, and 6.09×10^5, 5.96×10^5, and 2.53×10^6 copies g^{-1} in VFCW4, respectively. The results demonstrated that the absolute abundance of *nosZ*, *nirK*, and *nirS* genes in aeration systems was lower than that of the non-aeration systems. Aeration inhibits microbial heterotrophic denitrification due to the changes of anaerobic conditions (Yang et al. 2017). However, due to the addition of carbon sources, the copy number of *nosZ*, *nirK*, and *nirS* genes showed opposite results to the effects of aeration ($P < 0.05$). The probable reason was that supplementing the exogenous carbon source facilitated the growth of denitrifying bacteria, while the carbon limitation attenuated the denitrifying activity (Hou et al. 2018). Zhi and Ji (2014) recorded analogous conclusions in preceding studies. Furthermore, contrary to the research by Hu et al. (2016), a significantly higher absolute abundance of *nosZ* gene was obtained in this experiment, which means that complete denitrification may occur.

Relationship between N transformation and microorganisms

To further investigate the relationship between N transformation and microorganisms, an overview of the values of microbial compositions and functional genes was performed by RDA analysis. As Fig. 6 illustrated, the removal efficiency of COD, TN, NH_4^+-N, and NO_3^--N appeared unique distribution between microbial characteristics. The angles between N and functional genes suggested that *nosZ*, *nirK*, and *nirS* were mainly positively associated with NO_3^--N and COD removal, but they were negatively correlated with removals of NH_4^+-N. Moreover, *amoA* and *nxrA* were positively correlated with the removal efficiency of NH_4^+-N and were mainly

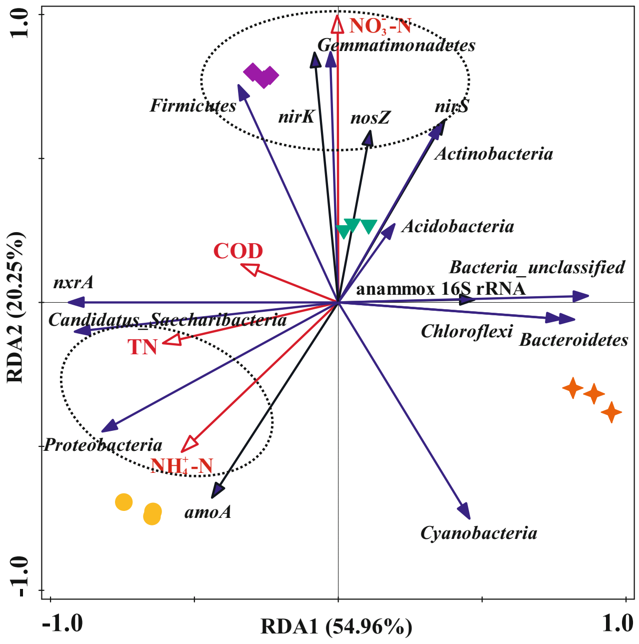

Fig. 6 The relationship between N removal and microorganisms revealed by redundancy analysis (down triangles represent VFCW1 with aerated and carbon source; circles represent VFCW2 with aerated and non-carbon source; diamonds represent VFCW3 with carbon source and non-aerated; stars represent VFCW4 with non-carbon source and non-aerated)

located in the aeration system VFCW2 (region represented by circles), indicating intermittent aeration promoted nitrification. Arrow position indicate that carbon source system VFCW3 (region represented by diamonds) was mainly associated with *Firmicutes* and *Actinobacteria*, which played a crucial role in heterotrophic denitrification process (Chen et al. 2017).

Conclusion

The combined impacts of intermittent aeration and exogenous carbon source addition in VFCWs significantly enhanced N and organic matter removal in the current study. Optimal average NH_4^+-N, TN, NO_3^--N, and COD removal efficiency (93.56, 86.88, 79.65, and 74.16%) were obtained in an aerated system accompanied by carbon source addition (VFCW1), which contained the highest richness and diversity of microbial community. The increase in the DO concentration and carbon source elevated the copy number of functional genes in microbial nitrification (*nxrA* and *amoA*) and heterotrophic denitrification (*nosZ*, *nirK*, and *nirS*), respectively. Overall, the appropriate combination of artificial intermittent aeration and exogenous carbon source addition is an effective operation to remove N and organics from the VFCW systems.

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