Impacts of feeding strategy on microbial community structure diversity in vertical flow constructed wetlands

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Abstract. The impacts of feeding strategy (intermittently or continuously) on contaminant removal performance and microbial community structure in vertical flow constructed wetlands (VFCWs) were evaluated. The results showed that intermittent feeding strategy improved the removal of COD, TP and ammonium in VFCWs, although TN removal was weakened correspondingly The bacterial diversity decreased with the increase of substratum depth in all CWs. The intermittent feeding favored the growth of microorganisms due to the enhancement of oxygen content in the substratum. The feeding strategy had little impact on the microbial community in the surface substratum. However, in the bottom substratum, the impacts were of great significance. The microbial community structure similarity between the CWs with different feeding strategies was low.

1. Introduction

In the present day, vertical flow constructed wetlands (VFCWs) have been widely developed in many countries to treat municipal wastewater, due to their high removal efficiency and low operation cost compared to other wetlands [1-3]. In the CWs, nutrients can be removed in alternate anaerobic and aerobic environments. However, the VFCWs are faced with problems during nitrogen removal, which are primarily caused by the low oxygen availability and incomplete nitrification in the substratum [4].

In order to improve the nutrient removal of VFCWs, many strategies have been studied in recent years, such as artificial aeration and step feeding [5]. Moreover, an intermittent feeding strategy has been proved as an effective method to enhance the nutrient removal in VFCWs [6-8]. The redox conditions of the wetland substratum could be improved by intermittent feeding, according to the operation of intermittent flooding and drainage. During the cyclic flood and drain operation, the

substratum is periodically exposed to the atmosphere, and the oxidation condition in the substratum is improved due to the fast oxygen diffusion [9]. It has been reported in previous literature that the removal efficiency of ammonium in intermittently fed horizontal subsurface flow CWs was 14% greater than that of traditional, continuously fed CWs [10]. Compared to artificial aeration, the intermittent feeding strategy is of low cost and simpler operation.

Microorganisms are considered to be responsible for the transformation and degradation of pollutants in CWs, and the living environment is important to the diversity of microbial community structure. Truu, et al., analyzed the distributions of the microbial community in a horizontal flow CW and found that the microbial community varied with bed depth and chemical properties [11]. In addition, it was found that bacteria were more abundant on root surfaces due to the better oxygen diffusion [12]. Since the intermittent operation can alter the redox condition of substratum, the microbial community structure would change correspondingly. However, few studies to date have focused on the impacts of feeding strategy on microbial community structure diversity in VFCWs.

In the present study, VFCWs were conducted and fed continuously or intermittently. The contaminant removal performance was determined and the microbial community structures were analyzed using the denaturing gradient gel electrophoresis (DGGE) technique. The aims of the present study are to evaluate the effects of feeding strategy on microbial community structure and to reveal the spatial distribution of bacterial communities in the VFCWs.

2. Materials and methods

2.1. Characterization of experimental system

The VFCWs used in this study were constructed in circular barrel polyethylene containers with surface areas of 0.24 m^2 and depths of 65 cm. The substratum consisted of three successive layers: at the bottom was a 5-cm layer of river gravel with particle diameter of 4-5 cm, and then a 40-cm layer was paved using gravel with particle diameter of 1-3 cm. At the top, washed sand was used with a depth of 15 cm. The CWs were fed with synthetic wastewater, and the complete influent contained 350 mg/L COD, 40 mg/L NH_4^+-N and 5 mg/L TP. The structure of the experimental system and the components of the synthetic wastewater can be found in previous literature [6].

Sequencing fill and the draw batch mode was employed in the CWs as the influent mode. The wastewater was pumped into the CWs within 10 minutes during the feeding period. The wastewater was then kept in the CWs for a certain number of days to be purified, and the effluent was then discharged from the bottom. In order to ensure the purification performance of the CWs, the hydraulic retention time was seven days. After the CWs were well-established, three different feeding strategies were applied to three systems. Two CWs (W1 and W2) were operated with the intermittent feeding mode and one (W3) was operated with the continuous feeding mode. For W1, the substratum was flooded for four days, the water was then discharged completely and the CWs were dried for three days to obtain an F/D ratio of 4:3 during one running cycle. For W2, the F/D ratio was 6:1. For W3, the substratum was flooded for seven days during one cycle. After running for approximately three months, the purification performance of all CWs was stable.

2.2. Sampling

The influent and effluent of all CWs was determined and then COD, ammonium, total nitrogen (TN) and total phosphorus (TP) concentrations were analyzed using the standard methods described in APAH-AWWA-WPCF [13].

The substratum in the upper and lower layers of all CWs were sampled using sampling apparatus, to investigate the microbial community structure at different layers. The sampling depths were 10-15 cm and 50-55 cm, respectively. In addition, in order to evaluate the impacts of fill and draw operation on microbial community structure, the sampling was conducted on the 4th and 7th days, respectively. The samples are listed in table 1.

Table 1. Sample list of VFCWs.

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
CWs	W1	W2	W3	W1	W2	W3	W1	W2	W3	W1	W2	W3
Layer	Upper			Lower			Upper			Lower		
Time	4th day						7th day					

2.3. Microbial analysis

The total genomic DNA from the substratum microbes of all samples was extracted using the DNA Isolation Kit. F357-GC clamp and R518 primers were used to amplify the bacterial 16S rDNA by touchdown polymerase chain reaction (PCR) [14], using the protocol described by Wu, et al., [15] which is described briefly as: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 65°C to 55°C for 50 seconds, and 72°C for 40 seconds; the first 20 cycles with 0.5°C decrease of the annealing temperature every cycle, and a final extension cycle at 72 °C for 10 minutes. DGGE analysis was then performed using the Bio-Rad Dcode system (Bio-Rad, USA). Electrophoresis was performed at 120 V for eight hours in 1×TAE buffer at a constant temperature of 60 ºC, and the liner gradient was 29-50%. In order to visualize the DGGE bands, the gels were stained by ethidium bromide and photographed under UV-light after electrophoresis.

2.4. Data analysis

The DGGE profiles were analyzed using Quantity One software (Bio-Rad, USA) to reveal the banding patterns. The diversity index and dominance index were then calculated, respectively [16, 17], according to the number of bands in each lane of the DGGE profiles. The richness (R) values of the microbial communities were represented by the total numbers of DGGE bands, and the evenness (E) was calculated according to the formula $E = H'/\ln R$. The Dice coefficient was calculated to analyze the community similarities, and cluster analysis was conducted and expressed by dendrograms, which was calculated by an unweighted pair group method with the arithmetic average (UPGMA).

3. Results and discussion

3.1. Impacts of feeding strategy on contaminant removal performance of VFCWs

The contaminant removal efficiencies of each wetland were determined and the results are shown in Figure. 1. It can be observed that the COD removal efficiency in all CWs exceeded 85%. However, the removal efficiencies of W1 and W2 were higher than that of W3, indicating that intermittent

feeding can improve the removal of COD. The intermittent feeding increases the oxygen content in the substratum, and the activities of aerobes are enhanced due to the alternant flooding and drying [6]. Moreover, the organic matter absorbed by the substratum can also be degraded during the drying period. Therefore, the organic matter is removed quickly in the intermittently-fed VFCWs. The removal of TP was also improved by intermittent feeding. It is possible that the physical-chemical accumulation processes, such as adsorption, precipitation or accumulation in the substratum were the dominant phosphorus removal processes [18]. The intermittent feeding supplied an alternate anaerobic-aerobic condition which was conducive to the metabolism of polyphosphate accumulating organisms (PAOs), and the phosphorus adsorbed by the substratum could be utilized by PAOs under aerobic conditions during the drying period of W1 and W2, and the adsorption capacity of the substratum would be correspondingly increased.
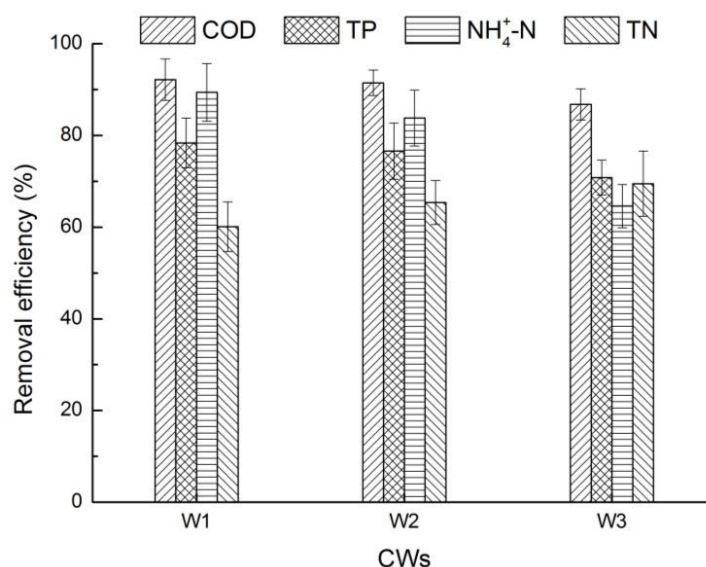

Figure 1. Contaminant removal efficiencies of the VFCWs.

It is noteworthy that the removal of ammonium was greatly improved by intermittent feeding. The NH_4^+-N removal efficiency of W1 and W2 was 30%-38% higher than that of W3. This is because the intermittent feeding increased the oxidization conditions in the substratum, thus facilitating the nitrification process. However, TN removal differed from that of ammonium, and the TN removal efficiency decreased as a result of intermittent feeding. In W1, the TN removal efficiency was only 60%, while it reached 69.5% in W3. Although some previous studied have reported that intermittent loading can increase TN removal [19, 20], this was not observed in the present study. It is thought that the denitrification process may be inhibited by the intermittent operation during the drying period.

3.2. Impacts of feeding strategy on bacterial communities diversity
The DGGE banding patterns of 16 S rDNA fragments of all samples are shown in figure 2. Results indicate that the DGGE patterns from CWs with different feeding strategies were distinct. The quantity of DGGE bands in the upper layers of all CWs (S1-S3 and S7-S9) was greater than that observed in the lower layers (S4-S6 and S10-S12), indicating that the bacteria species in the upper layers were more abundant than those in the lower layers for all CWs. Some bands, such as band 1 and

2, were commonly detected in all CWs, suggesting that microbes with these bands were stable, and were not affected by the feeding strategy. However, some bands also emerged or vanished with individual CWs. Band 3 emerged in the upper layer of W2 and W3 with longstanding flooding. However, band 5 appeared in both the upper and lower layers of W1, and vanished in W2 and W3. These results indicate that the different feeding strategies changed the living environment of microbes, and that some bacteria were eliminated due to their inability to adapt to changes.
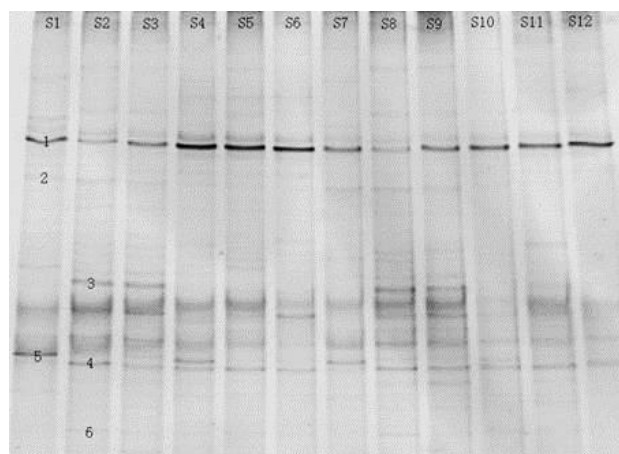
Figure 2. DGGE profiles of 16S rDNA from CWs with different feeding strategies.

Table 2. The bacterial community richness, diversity index, community dominance and evenness from different CWs.

Samples	R	H'	D	E
S1	26	3.11	0.05	0.95
S2	24	2.66	0.08	0.84
S3	28	2.67	0.09	0.80
S4	14	1.09	0.46	0.41
S5	17	1.07	0.49	0.38
S6	12	0.69	0.66	0.28
S7	15	2.47	0.09	0.91
S8	25	2.64	0.10	0.82
S9	24	2.21	0.16	0.70
S10	12	1.67	0.23	0.67
S11	19	1.69	0.26	0.57
S12	11	1.06	0.40	0.48

The richness, diversity, dominance and evenness of bacterial communities in CWs with different feeding strategies were calculated based on the DGGE patterns, and the results are shown in table 2. It was presumed that each band in the DGGE profiles represented one phylogenetically distinct population. Therefore, R was equal to the total numbers of the bands, and ranged from 11 to 28 bands per lane during the operation. Results indicate that the indices of H' and E in the upper layers were

significantly higher than those observed in the lower layers, indicating that the species of microorganisms in the upper layers of all CWs were more abundant. Alternatively, the dominance indices (*D*) of the lower layers were greater. In the subsurface flow CWs, the pollutant would be held and adsorbed first by the surface substratum, which is more conducive to the growth of microorganisms [12]. As the depth increased, the organic matter and nutrients in the substratum decreased gradually, and some microorganisms would be eliminated resulting in correspondingly lower diversity.

As shown in table 2, the diversity in the upper layers of W1 on day four was much higher than that observed in W2 and W3 (S1 vs. S2 and S3). Moreover, indices of H' and E in the lower layers of W1 and W2 were significantly greater than that of W3. These results indicate that intermittent feeding favored the growth of microorganisms in both the lower and upper layers due to the enhancement of oxygen content in the substratum.

Table 3. Dice coefficient within and between samples of CWs with different feeding strategies.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
S1	1											
S2	0.754	1										
S3	0.722	0.710	1									
S4	0.499	0.472	0.484	1								
S5	0.477	0.524	0.528	0.629	1							
S6	0.476	0.536	0.505	0.594	0.655	1						
S7	0.496	0.528	0.517	0.508	0.418	0.451	1					
S8	0.507	0.498	0.518	0.432	0.578	0.427	0.559	1				
S9	0.547	0.524	0.502	0.390	0.564	0.412	0.574	0.741	1			
S10	0.405	0.366	0.382	0.460	0.507	0.447	0.224	0.326	0.318	1		
S11	0.578	0.639	0.602	0.539	0.493	0.525	0.553	0.458	0.412	0.262	1	
S12	0.373	0.460	0.428	0.481	0.382	0.525	0.524	0.305	0.259	0.277	0.683	1

Dice coefficients were calculated based on the DGGE profiles to reveal the similarity among all samples, and the results are shown in table 3. Moreover, cluster analysis based on the Dice coefficients was conducted and expressed byvisualized dendrograms to investigate the community similarity among different wetlands (figure 3). Results indicate that the Dice coefficient of all samples varied greatly. The similarity coefficient of the upper-layer samples was greatest, indicating that the feeding strategy had little impact on the microbial community in the surface substratum. The similarity between the upper samples and lower samples was low, mainly caused by the differing oxygen content. In the surface substratum, the oxidizing condition favored the existence of aerobes. However, the bottom substratum it was mainly reductive, where amphimicrobes and anaerobes are the dominant microorganisms. The S5 and S6 samples demonstrated high community similarity (65%), and the similarity between S4 and S6 was low (figure 3). This is because W1 had a longer drying period than W2, and the bottom substratum of W1 could maintain oxidizing conditions. Although intermittent feeding was also used in W2, the F/D ratio was 6:1, and the drying time was only one day. Therefore, the similarity between the bottom substratums of W2 and W3 was higher. It is noteworthy that the

samples taken on days four and seven had different coefficients. This may be because on day seven, W1 has been dried for three days, and some biofilm existing in the substratum had decayed when sampling. Therefore, the coefficient varied.
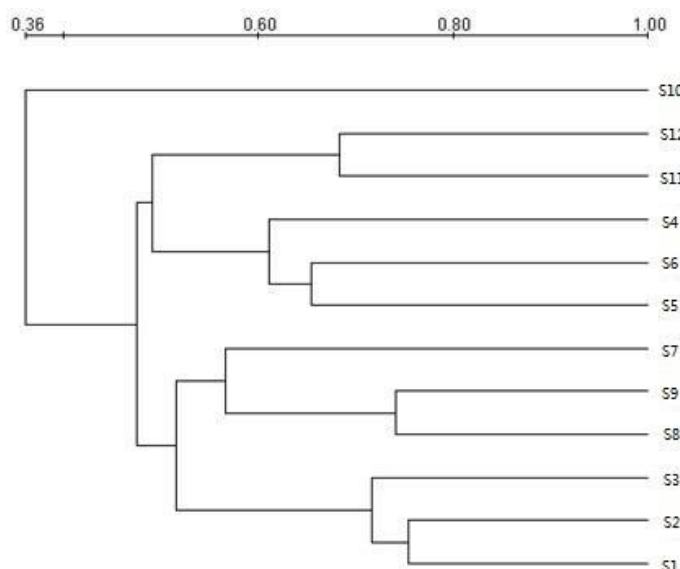
Figure 3. Cluster analysis of the 16S rDNA DGGE patterns.

4. Conclusions

Intermittent feeding canenhance the removal of COD, TP and ammonium in VFCWs. However, TN removal was weakened by intermittent feeding due to the inhibition of denitrification. A decrease in bacterial diversity with increasing substratum depth was found in all CWs, independent of which feeding strategy was used. In the substratum, the growth of microorganisms was enhanced by intermittent feeding due to the long drying time. The diversity in the upper layers of W1 was much greater than that observed in W2 and W3. In the bottom substratum, the feeding strategy had significant impacts on microbial community. The microbial community structure similarity between the CWs with different F/D time ratios was low.

References

[1]   Brix H and Arias C A 2005 *Ecol. Eng.* **25** 491-500
[2]   Wu H, Zhang J, Li P, Zhang J, Xie H and Zhang B 2011 *Ecol. Eng.* **37** 560-8
[3]   Zheng Y, Wang X, Dzakpasu M, Zhao Y, Ngo H H, Guo W, Ge Y and Xiong J 2016 *Bioresource Technol.* **207** 134-41
[4]   Brix H, Arias C A and del Bubba M 2001 *Water Sci. Technol.* **44** 47-54
[5]   Kirui W K, Wu S B, Kizito S, Carvalho P N and Dong R J 2016 *J. Environ. Manage.* **166** 38-44
[6]   Jia W, Zhang J, Wu J, Xie H and Zhang B 2010 *Desalination* **262** 202-8
[7]   Jia W, Zhang J, Li P, Xie H, Wu J and Wang J 2011 *Ecol. Eng.* **37** 1815-21
[8]   Wu H, Fan J, Zhang J, Ngo H H, Guo W, Hu Z and Liang S 2015 *Bioresource Technol.* **176** 163-8
[9]   Behrends L, Houke L, Bailey E, Jansen P and Brown D 2001 *Water Sci. Technol.* **44** 399-405
[10]   Caselles-Osorio A and Garcia J 2007 *Sci. Total. Environ.* **378** 253-62

[11]   Truu J, Nurk K, Juhanson J and Mander U 2005 *J. Sci. Environ. Heal. A* **40** 1191-200

[12]   Wang Q, Xie H, Ngo H H, Guo W, Zhang J, Liu C, Liang S, Hu Z, Yang Z and Zhao C 2016 *Environ. Sci. Pollut. R.* **23** 4036-45

[13]   APAH-AWWA-WPCF 2001 *Standard methods for the examination of water and wastewater* 20th ed. (Washington: American Public Health Association)

[14]   Muyzer G, Dewaal E C and Uitterlinden A G 1993 *Appl. Environ. Microb.* **59** 695-700

[15]   Wu J, Zhang J, Jia W, Xie H and Wu H 2009 *Int. Conf. on Environmental Science and Information Application Technology* (Wuhan: China) pp 160-163

[16]   Shannon C E and Weaver W 1963 *The mathematical theory of communication* (Illinois: The University of Illinois Press)

[17]   Simpson E H 1949 *Nature* **163** 688

[18]   Moortel A M K, Rousseau D P L, Tack F M G and de Pauw N 2009 *Ecol. Eng.* **35** 175-83

[19]   Merlin G, Pajean J L and Lissolo T 2002 *Hydrobiologia* **469** 87-98

[20]   Li Y, Li H, Sun T and Wang X 2011 *J. Hazard. Mater.* **189** 336-41