Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2017.21577

94 (2017) 56–63
October

Performance and the microbial community of horizontal subsurface-flow constructed wetland for rural sewage treatment

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Received 20 December 2016; Accepted 23 October 2017

ABSTRACT

Horizontal subsurface-flow constructed wetlands are commonly used to remove pollution from sewage. This study compared the treatment performance and microbial community structure of wetland with *Phragmites communis*(A) and *Typha orientalis Presl*(B) in different seasons. Two parallel pilot-scale wetlands for rural sewage treatment were operated for one year. The average removal efficiency of pollutants from high to low is in the order of summer > spring > autumn > winter, and the highest removal rates for COD, NH_3-N, and TP are 90, 92, and 69.29%, respectively. Pollutant concentrations are lower in wetland B than those of wetland A, thereby indicating that the purification capacity is better in *Typha orientalis Presl* than *Phragmites communis*. The microbial community of the wetland was analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis. This analysis showed that the numbers of dominant bands are greater and relatively brighter in spring and summer than in autumn and winter. The presence of least bands during winter indicates that the structure of microorganisms is relatively simple at low temperature. The highest and lowest Shannon indexes are in summer and winter, respectively. Results of the sequencing analysis show that *Firmicutes*, *Bacteroidetes* and *Proteobacteria* are the three microbial communities with the highest pollutant removal rate.

Keywords: Rural sewage; Wetland; Seasons; Plants; Microbial communities

1. Introduction

Rural sewage plays a crucial role in non-point source pollution, and rural sewage treatment has been extensively adopted. Constructed wetland, which is characterized by low cost, convenient management, stable treatment, and suitability for sewage treatment in rural areas,is composed of porous materials, plants, and microorganisms [1]. Sewage in horizontal subsurface-flow wetland is flows slowly under filling layers. The continuous release of oxygen in plant roots consequently causes aerobic, anoxic, and anaerobic effects [2]. In addition, sewage below the filling layers can also prevent the breeding of mosquitoes. Porous material is an important carrier of wetlands, generally by single or mixed filler (soil, sand, gravel and cinder) with different proportions, which support plant growth and provide attachment points for microorganisms [3]. Microorganisms such as fungi, bacteria, protozoan, phytoplankton, and zooplankton are mainly distributed on the surface of filling layers and plant roots [4]. Constructed wetland is not only a collection of porous materials, plants, and microorganisms, but also a complex artificial ecosystem with suitable configuration and combination.

The influence of plants on purifying pollutants is closely related to plant species and developing a root system [5,6]. Thus, the purification efficiency of different plants is evidently different. Four main kinds of plants grow in wetlands: aquatic, floating, floating-leaved, and submerged macrophytes [7]. The current study focused on bacterial and archaeal communities with different plants. The plant type significantly influenced the microbial community [8]. Cristina investigated the application of different plant species in wetlands [9]. A consensus on

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the differences among plant species has not been reached, and several results are based on specific experimental conditions. The same plant under different experimental conditions may even yield an opposite conclusion. Plant species affect the distribution of microbial communities in wetlands, thereby resulting in varying pollutant removal rates. The plant–microbe–pollutant interaction in wetlands requires investigation. In spring and summer, plants grow vigorously; thus the demand for nutrients and the plant absorption are significantly high [10]. In addition, the seasonal variation affects the photosynthesis of plants, which affect the transport of oxygen and organic matter near the roots. The most important participants in purification are microbes. Temperature has a significant impact on the number of microorganisms, community structure, and activity. The nitrification of microorganisms was stable at 10 °C and decreased significantly to lower than 10 °C [11]. Nitrification rate declined rapidly to zero at 6 °C and denitrification rate was also low at 5 °C; this scenario indicates that the temperature can directly affect the removal efficiency of pollutants in wetlands. In summer, various biochemistry reactions are evident because the temperature is high. Therefore, temperature is an important factor to consider in designing constructed wetlands. Traditional research on for microbial diversity involves isolation, culture and identification of microbial strains by pure culture technique [12]. However, 85–99% of microorganisms cannot be cultured. Culture in this case may lead to severe underestimation of microbial diversity. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has been extensively applied in research on microbial diversity in constructed wetlands to overcome the limitations of traditional methods [13]. Truu studied the microbial community in horizontal subsurface-flow constructed wetland using PCR-DGGE [14]. Li performed sequence analysis by cloning gene technology, and the microbial community near the root was obtained [15]. PCR- DGGE results [16] showed that the Shannon diversity index decreases significantly from influent to effluent, as confirmed by Nie [17].

It is significant to study the effect of seasonal variation and plant type on pollutant removal. In view of this, the removal efficiencies in two constructed wetlands (A and B) are investigated. The mechanisms of microorganism on the removal of pollutants is deeply discussed by PCR-DGGE, which can provide theoretical references for further study on the mechanism of pollution purification and the optimization designing wetlands.

2. Materials and methods

2.1. Construction of the constructed wetlands

Fig. 1 presents the schematic diagram of subsurface-flow constructed wetlands, which was operated in Nanjing, Jiangsu Province, with average temperature of 15.4 °C and annual rainfall of 1200 mm. On the whole, four seasons have their characteristics: spring and autumn is short, winter is cold and dry, while summer is hot and rainy. The wetland system was made of polyvinyl chloride, with length, width and depth of 130, 50 and 60 cm, respectively. Domestic wastewater was stored in a plastic cistern and transferred into the apparatus by a pump. Plants and microbial communities should be domesticated for three months before the test data are collected. Sampling points along the constructed wetlands were labeled S1, S2, S3 and S4, with the same horizontal distance of 40 cm. S1 and S4 are only wastewater, because they are influent and effluent, respectively. For S2 and S3, the samples are the mixture of water and substrate, so that the analysis is more comprehensive. The sequential layers of material in system A from bottom to top were: larger gravel (diameter of 30–50 mm, height

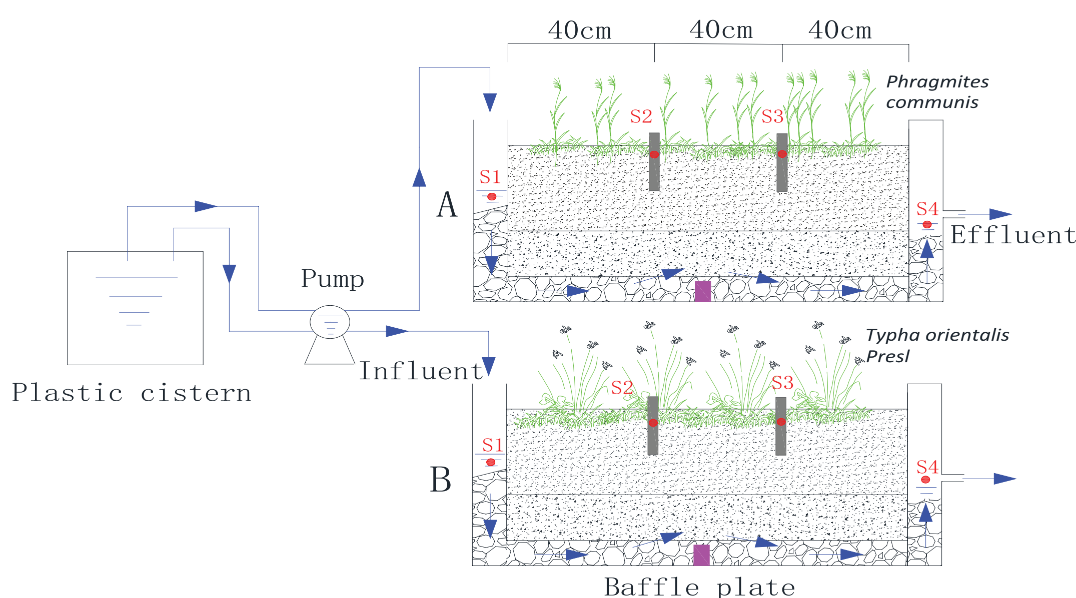

Fig. 1. Schematic diagram of horizontal subsurface-flow constructed wetlands: A- *Phragmites communis; B- Typha orientalis Presl.*

of 80 mm), slag-coal (diameter of 10–30 mm, height of 150 mm) and artificial soil (nutrient soil rich in nitrogen and phosphorus, height of 300 mm). *Phragmites communis* were planted at a density of 20–30 strains per square meter, and there was 14–20 strains in wetland A. Filling materials of equipment B were the same as A, with different plants (*Typha orientalis Presl*).

2.2. Water quality analysis

The influent of wetlands is artificial sewage as shown in Table 1, which was added with $(NH_4)_2SO_4$, glucose, peptone and KH_2PO_4. The average daily inflow is 160 L with hydraulic retention time (HRT) of 2.25 d. The hydraulic surface load was maintained at 0.40 m^3/ (m^2·d).

Influent and effluent of subsurface-flow wetlands were collected and analyzed at around 10 am, 2–3 times per week. There were also two horizontal sampling points along the wetlands, with distance of 40 cm. The chemical oxygen demand (COD), ammonium nitrogen (NH_4^+-N) and total phosphorus (TP) were determined according to standard methods (APHA 2005). Demand oxygen was assayed by an Orion Dissolved Oxygen Probe (Model 862 A plus, USA) and pH was measured using an Orion Portable pH Meter (Model 250 A plus, USA). All data were conducted in triplicate. The removal efficiency was calculated as $\eta = (1 - \omega_i / \omega_e) \times 100\%$, where w_i and w_e are the influent and effluent concentration, respectively.

Table 1
Characteristic of the influent

pH	6.49–7.16
DO (mg/L)	1.2–2.3
COD (mg/L)	150–215
TP (mg/L)	6.12–10.15
NH_3-N (mg/L)	21.63–39.00

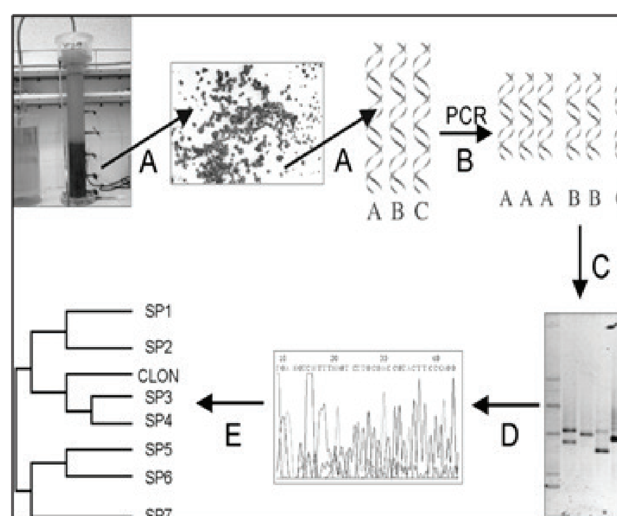

Fig. 2. The main procedures of PCR-DGGE.

2.3. Microbial analysis

As shown in Fig. 2, the analysis of microbial community by PCR-DGGE mainly includes the following steps [18]. Firstly, the samples were pretreated and DNA was extracted in step A, following by two times PCR with specific primers in procedure B. Then the quantified bacteria were processed by DGGE as the program C. The dominant bacterial bands on DGGE graph were recovered, purified and amplified again for sequence analysis by the Basic Local Alignment Search Tool in step D. Finally, according to the principle of maximum homology, the compared results by Clustal software were input Molecular evolutionary genetics analysis (MEGA) to establish neighbor-joining tree.

Samples of influent, effluent, and sampling tunes in Fig. 1 were obtained. The Ultra Clean™ Soil DNA was used to study microbial organisms. They were added to a bead beating tube containing beads, lysis solution and inhibitor removal solution. Released DNA was bound to a silica spin filter. The filter was washed, and the DNA was recovered as the template for PCR. The first PCR was conducted with two primers 63F:CAGGCCTAACACATG-CAAGTC and 1387R: GGGCGGWGTGTACAAGGC. The initial condition was 10 min at 94 °C for enzyme activation and the following steps for 30 cycles: 45 s at 94 °C, 60 s at 55 °C and 45 s at 72 °C. After the cycle is over, the sample was kept at 72 °C for 10 min. The first PCR products were used as the template in the second PCR, with two primers338F-GC:CGCCCGCCGCGCGCGGCGGGCGGGGC-GGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG and 518R:ATTACCGCGGCTGG. The reaction also includes 30 cycles of 60 s at 95 °C, 120 s at 63 °C and 60 s at 72 °C. 10% polypropylene acyl gel was prepared with denaturing agent concentration from 50% to 80%. The concentration of denaturing agent and acrylamide was increasing from top to bottom. After gel was completely polymerized, the glue board was put in the electrophoresis tank, and then 5 µl PCR sample and buffer were add into the sample hole. DGGE was performed at voltage of 100 V for 14 h. The samples were dyed 15 min with ethidium bromide, decolorization 25 min and then took pictures. The dominant bands in DGGE gel were excised and amplified again by PCR. Finally, the amplified DNA fragments were sent to the Shanghai Boya biotechnology company for sequencing.

Analysis software Quantity One was used to analyze the DGGE spectra and the diversity index of Shannon was applied to evaluate the microbial diversity of the samples. The diversity index was derived from the long-term experiment, and the formula was:

$$H = -\sum_{i-1}^{s} Pi \log P1 = -\sum_{i-1}^{s} (ni / N \log(ni / N) \quad (1)$$

In the formula, n_i is the peak area, and N is the total area of all peaks.

3. Results and discussion

3.1. COD removal

The removal of organic matter in constructed wetlands depends mainly on the absorption and biological metabo-

lism of microorganisms on substrate and plant roots. The presence and activity of microorganisms directly affect the treatment effect of the system, and the variation of plant growth caused by seasonal changes also slightly influences the treatment effect.

According to the general trend in Fig. 3, the removal efficiency of chemical oxygen demand (COD) in systems A and B from high to low was in the order of summer > spring > autumn > winter. The fluctuation of the treatment effect of COD was volatile as months passed. The removal efficiency of COD from September 2015 to July 2016 first decreased and then increased. The main difference across the months was the temperature. The external temperature from September to December gradually decreased from above 30 °C to approximately 0 °C. Low temperature weakened the activity of microorganisms, and the removal efficiency of COD decreased. In January, the temperature dropped to the minimum; the device nearly stopped working during this period. The figure demonstrates that the COD removal rate can be maintained at more than 80% throughout the year despite the poor external conditions. Analysis of the reasons revealed that the winter temperature weakened the microbial activity on the surface of wetlands, but the microbial activity in the interior remained high in protecting the outer microbes. The total numbers of microorganisms of different species are small. Only favorable environmental conditions can promote microbial reproduction and growth.

The pollutant concentration in effluent does not change significantly. This study considers the monitoring data of May as an example for the analysis. Fig. 4 illustrates the COD variation along the length of the wetland in systems A and B. In this figure the COD concentration of the influent had a certain fluctuation, but the effluent was lower than 20 mg/L, which is stable enough to reach the first level of GB18918-2002 municipal wastewater discharge standards [19]. This condition demonstrates that the subsurface-flow constructed wetland has a high capacity for COD load. Organic matter in the front 1/3 of wetland is effectively removed, and that of the subsequent 2/3 segment is relatively small. The main reason is that the microorganisms and plants in the former segment breed rapidly under adequate nutritional conditions and thus maintain a high removal rate of organic matter [20].

3.2. NH_3-N removal

Fig. 5 presents the removal effect of ammonia nitrogen (NH_3-N) by month. The removal of NH_3-N and COD with seasonal variation is the same that is, from high to low, summer > spring > autumn > winter. The removal efficiency of NH_3-N is considerably better in system B (*Typha orientalis Presl*) than system A (*Phragmites communis*). In summer, the average removal rate of NH_3-N can reach more than 80%. A high temperature is beneficial to plant photosynthesis, because additional oxygen is generated and transmitted to the plant root and forms a series of aerobic and anaerobic environments. This is conducive to nitrification and denitrification. Besides, high temperature is also beneficial to the volatilization of NH_3-N. The plant growth in spring has strengthened the absorption of NH_3-N, although the temperature difference in spring and autumn is not evident. Thus, the removal rate is higher in spring than in

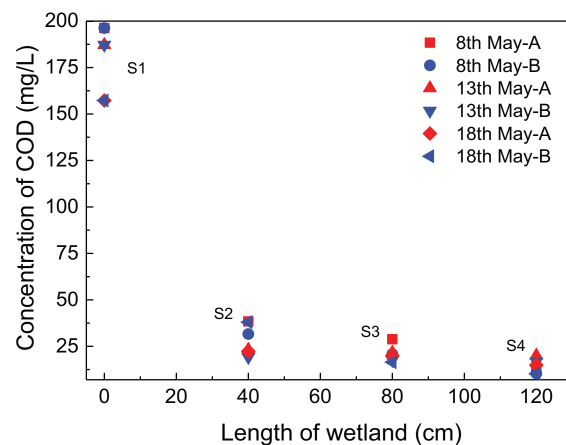

Fig. 4. Variation of the removal performance of COD.

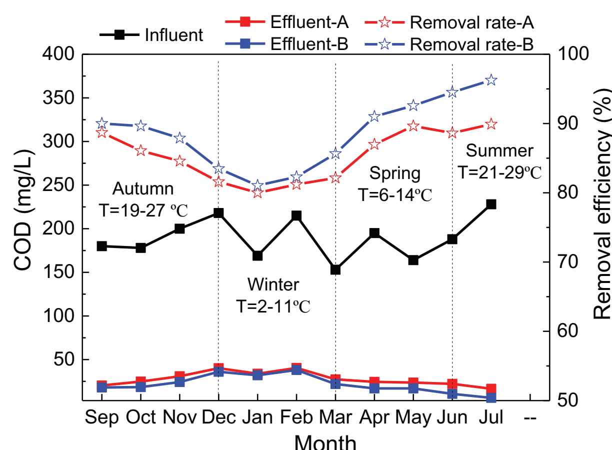

Fig. 3. Variation of the removal performance of COD in systems A and B.

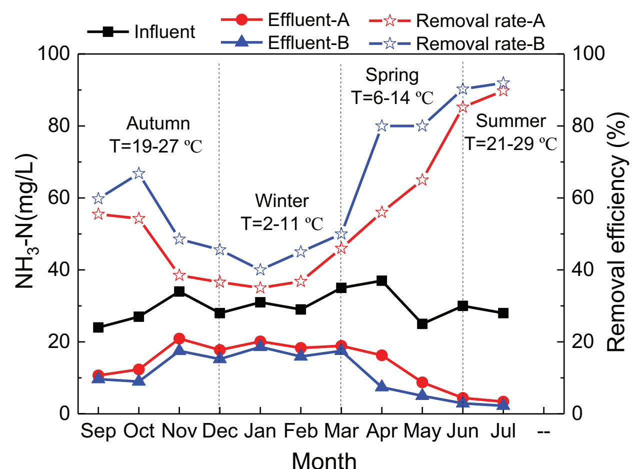

Fig. 5. Variation of the removal performance of NH_3-N in systems A and B.

autumn. The bacterial activity in the wetland is low and its metabolism is slow given the low temperature during winter. Simultaneously, the plants withered into the dormant period, oxygen supply capacity was limited, and nitrogen demand was low. Thus, the removal rate of NH_3-N was less than 50% and decreased by 15–20% compared with that of autumn.

In Fig. 6, the NH_3-N concentration in constructed wetlands gradually decreased during the process; this activity is similar to the degradation of COD. In the front 1/3 of the wetland, the pollutant removal was evident. Simultaneously,the NH_3-N concentration of each sampling point was evidently lower in system B than system A. This condition indicates that the denitrification capacity of NH_3-N in system B is higher. This conditionis mainly due to *Typha orientalis Presl* has a developed root system, and its oxygen capacity and microbial activity is robust. Thus, the nitrification and denitrification capacities are significantly in *Typha orientalis Presl* than in *Phragmites communis*.

3.3. TP removal

Fig. 7 depicts that the highest removal rate of TP is during summer. The removal efficiency of TP during spring and autumn ranges from 40% to 60%. The removal effect in winter decreased by approximately 15% compared with the removal effect during these periods. The reason for this difference is mainly related to the TP removal mechanism in wetlands. The TP in wetlands is mainly absorbed by plants and the adsorption of filling layers. The plant growth statuses during spring and autumn are similar, and the removal level of TP is not quite different. Plants grow vigorously in summer, which promotes root growth, thus enhancing the water absorption by roots. The highest TP removal rate is observed in summer given the increased activity of microorganisms. In winter, the temperature is usually below 10 °C, and plant growth is slowed down, thus affecting the absorption of TP in sewage. The removal rate of TP is lowest in winter, only approximately 40% because plant degradation can also release phosphorus-containing compounds. Therefore, the removal efficiency of TP in different seasons from high to low is in the order of summer > spring > autumn > winter.

Similarly, the TP concentration is lower in wetland B than in wetland A, thereby indicating that wetland with *Typha orientalis Presl* has better removal effect. The reason for this condition may be related to P biogeochemical cycles, such as plant uptake and microbial action, because the filling materials in the two kinds of wetlands are the same. The present study also demonstrated that different plant species have significant differences in phosphorus adsorption. The removal principles of TP in the wetland system include the assimilation of microorganisms, absorption of plants, P-uptake of the denitrifying phosphorus bacteria, and absorption of materials. The most important principle is the absorption capacity of TP in materials. Fig. 8 validates this finding. The TP concentration gradually decreases along the length. However, the magnitude of TP reduction in different locations is similar, because of the absorption of filling materials in wetlands.

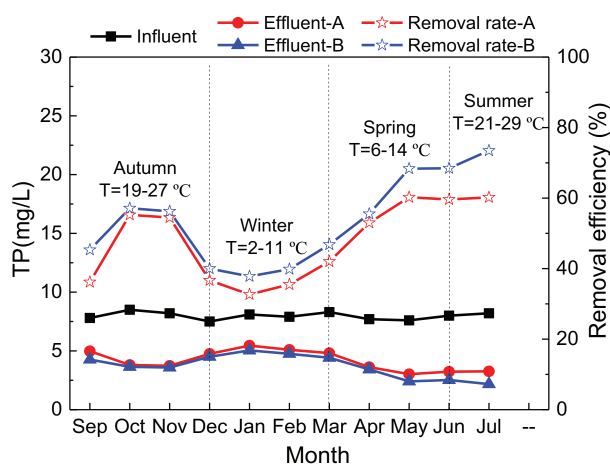

Fig. 7. Variation of the removal performance of TP in systems A and B.

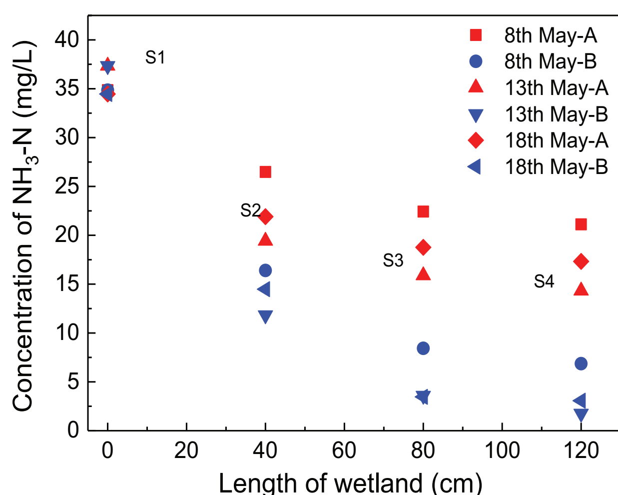

Fig. 6. Variation of the removal performance of NH_3-N.

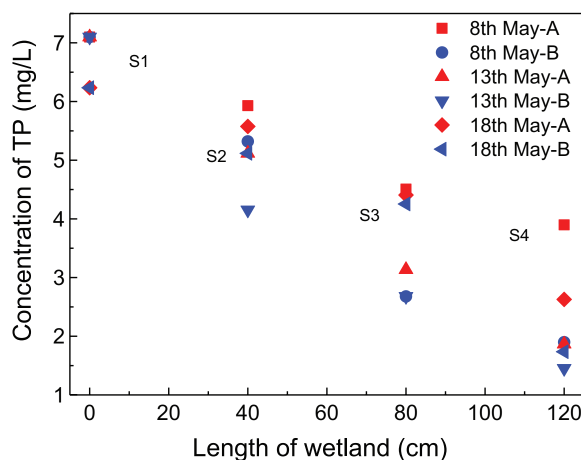

Fig. 8. Variation of the removal performance of TP.

3.4. Microbial community

The DGGE bands of different seasons are presented in Figs. 9 and 10. Spring and summer exhibit more dominant bands of wetland than autumn and winter. The results show that the microbial species are more abundant in spring and summer than in winter and autumn; this condition is one of the reasons for the enhanced treatment effect of pollutants. The number of dominant bands, especially in the winter of 2015, is the lowest, which is consistent with the worst treatment effect.

The typical bands in the influent (S1), which are not found in the samples of the wetland (S2, S3, and S4) in autumn and winter (Fig. 9) are 1, 2, 8, 9, and 11. This finding indicates that the community structure of the microorganisms changed because the sewage enters into the wetland system. The present and relatively clear bands in the two wetland systems (A and B) are 10, 12 and 14. The typical bands in the influent (S1), which are not found in the samples of the wetland (S2, S3 and S4), in spring and summer (Fig. 10) are 21, 22, 23, 25, 27, 30, 33, 35, 37, 38, and 42. The dominant species in summer and spring are 28, 24, 29, 31, 32, 34, 36, 40, and 41 bands. No specific bands are found in each season, and the microbial species of the two wetland systems (A and B) are small. Simultaneously, the brightness of the different samples at the same location evidently varies. All the above findings show that the microorganisms have a certain difference.

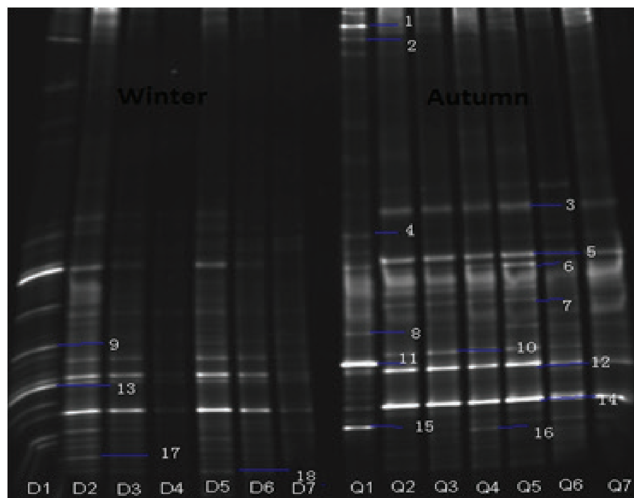

Fig. 9. The profile of DGGE bands in autumn and winter (D-Winter, Q-Autumn; 1-S1, 2-S2(A), 3-S3(A), 4-S4(A), 5-S2(B), 6-S3(B),7-S4(B)).

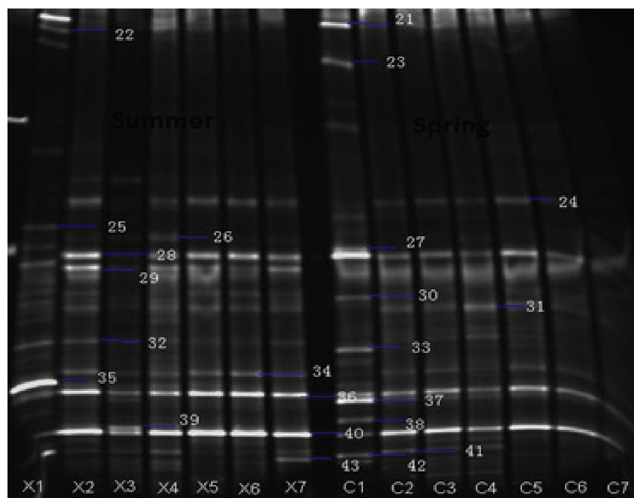

Fig. 10. The profile of DGGE bands in spring and summer (X-Summer, C-Spring; 1-S1, 2-S2(A), 3-S3(A), 4-S4(A), 5-S2(B), 6-S3(B),7-S4(B)).

The Shannon index of the DGGE analysis from the two wetland systems is listed in Table 2. The Shannon index of every sample shows an increasing and then a decreasing trend with seasonal variation, thereby indicating that the microbial diversity increases first and then decreases. Among the samples, the Shannon index in summer is largest and has the best treatment for pollutants. In winter, the Shannon index of B-S2 and A-S2 are not the lowest, and their microbial diversities remain high. However, the removal efficiency of pollutants is the minimum, thus indicating that the removal efficiency of pollutants is related to the activity of microorganisms. In a wetland system, the Shannon index decreases gradually in the direction of wastewater flow. This condition is associated with a gradual decrease in pollutant concentration and microorganism growth and propagation. Each sample of wetland nearly has higher Shannon index with *Typha orientalis Presl* than the *Phragmites communis* system by comparing the two systems.

The dominant bands in DGGE are recovered, amplified by PCR, and used for sequencing analysis. Unlike the sequences in the database, the maximum similarity sequence information (Appendix 1) is obtained. The homology analysis of the 41 bands (1–18 in autumn and winter; 21–43 in spring and summer) reveals that the most similar bacteria of Bands 6 and 29, 9 and 33, 11 and 35, 14 and 34, and 40 are the same. The attachment shows that most of the similar bacteria of dominant bands are uncultured. The dominant bands of the DGGE profile are mainly divided into five species, namely, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Chlorobi*, and *Cyanobacteria* through MEGA and CLASTALX software to construct a phylogenetic tree. In Table 3, the main microorganisms are *Firmicutes* and *Proteo-*

Table 2
Variation of the Shannon index

	Spring	Summer	Autumn	Winter	Average
B-S1	0.99778	0.99944	0.99811	0.99841	1.0484
B-S2	0.99967	1.07577	0.95256	0.99931	1.0068
B-S3	0.99933	1.04072	0.90191	0.69879	0.91019
B-S4	0.90254	1.04060	0.90174	0.47813	0.83150
Average-B	0.97483	1.0391	0.93858	0.79441	–
A-S1	0.99778	0.99944	0.99811	0.99841	1.0484
A-S2	0.84489	0.99925	0.95359	0.95378	0.93788
A-S3	0.77793	0.95388	0.95314	0.69877	0.84593
A-S4	0.60194	0.90246	0.90283	0.47711	0.72108
Average-A	0.80563	0.96376	0.80572	0.78202	–

Table 3
Microbial populations in different seasons

Seasons species	Spring	Summer	Autumn	Winter
γ-proteobacteria	4	4	1	–
β-proteobacteria	3	2	1	3
Bacteroidetes	3	5	4	–
Chlorobi	–	1	1	2
Cyanobacteria	–	–	1	1
Firmicutes	4	4	7	7

bacteria in winter, while several microbes belong to *Chlorobi*. In autumn, the most important microorganisms are *Firmicutes* and *Bacteroidetes*. In spring and summer, the dominant populations are mainly *Firmicutes, Bacteroidetes*, and *Proteobacteria*, with relatively average distribution. Therefore, the main aim of pollutant removal is the three types of bacteria, namely, *Firmicutes, Bacteroidetes,* and *Proteobacteria*.

4. Conclusions

The removal efficiency of pollutant from high to low is in the order of summer > spring > autumn > winter, and the highest removal rates for COD, NH_3-N and TP are 90, 92 and 69.29%, respectively. COD and NH_3-N decrease along the length of system, which are in accord with the running mode of the push flow. TP concentration in wetlands is gradually reduced, and the decrease range is uniform and stable. Pollutant concentrations in wetland B are lower than those of wetland A, indicating that the purification capacity is better in *Typha orientalis Presl* than *Phragmites communis*. PCR-DGGE results demonstrate that the number of dominant bands in spring and summer are more than that of autumn and winter. Shannon index is the highest and lowest in summer and winter, respectively. Along the direction of stream, Shannon index decreases gradually. The results of the sequencing analysis show that the dominant microbes are mainly divided into five species, namely, *Proteobacteria, Bacteroidetes, Firmicutes, Chlorobi*, and *Cyanobacteria*. The three most important microorganisms are *Firmicutes, Bacteroidetes* and *Proteobacteria*.

Acknowledgments

This research is supported by Scientific Research Foundation of Graduate School of Southeast University (YBJJ1644).

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Appendix 1

Sequencing results

Band	Most similar bacteria	Classification	Accession code	Similarity
1	Uncultured Bacteroidales bacterium clone HK6	*Bacteroidetes*	EU573848.1	100%
2	Uncultured Bacteroidetes bacterium clone SS69	*Bacteroidetes*	HM442633.1	100%
3	Uncultured Trichococcus sp. gene, clone: TCE-18	*Firmicutes*	AB529554.1	100%
4	Uncultured Bacteroides sp. clone J3	*Bacteroidetes*	DQ168847.1	100%
5	Uncultured Trichococcus sp. gene, clone: TCE-141	*Firmicutes*	AB529654.1	99%
6	Uncultured Bacteroidetes bacterium clone TH_a140	*Bacteroidetes*	EU373113.1	99%
7	Uncultured Trichococcus sp. gene, clone: TCE-109	*Firmicutes*	AB529627.1	98%
8	Acinetobacter sp. R-3(2010)	γ-proteobacteria	HM069069.1	92%
9	Oxalobacteraceae bacterium enrichment culture clone MWE_C40	β-proteobacteria	FJ391493.1	95%
10	Trichococcus sp. enrichment culture clone MB1_2	*Firmicutes*	AM933652.1	95%
11	Uncultured Chlorobium sp. clone 4.14	*Chlorobi*	GQ183422.1	99%
12	Uncultured Trichococcus sp. gene, clone: TCE-58	*Firmicutes*	AB529586.1	99%
13	Uncultured Thermosynechococcus sp. clone DTB123	*Cyanobacteria*	EF205530.1	86%
14	Trichococcus sp. 93	*Firmicutes*	EU325618.1	100%
15	Uncultured Curvibacter sp. clone REG_R2P4_E4	β-proteobacteria	FJ933337.1	99%
16	Uncultured Firmicutes bacterium gene ,isolate: DGGE band: 5j	*Firmicutes*	AB541164.1	100%
17	Uncultured Chlorobium sp. clone i9-117	*Chlorobi*	DQ383315.1	99%
18	Uncultured beta proteobacterium, clone B-LO-T0_OTU9	β-proteobacteria	FM204950.1	98%
21	Pseudomonas sp. Iw-1 Pseudomonas sp. PcFRB090	γ-proteobacteria	AB374923.1 AB569955.1	100% 99%
22	Uncultured Bacteroidetes bacterium clone SS834	*Bacteroidetes*	HM442357.1	100%
23	Pseudomonas sp. PcFRB101 gene	γ-proteobacteria	AB548838.1	97%
24	Trichococcus collinsii, strain 37AN3	*Firmicutes*	AJ306612.1	99%
25	Uncultured Bacteroides sp. clone L26	*Bacteroidetes*	GQ332238.1	99%
26	Uncultured Bacteroidetes bacterium clone 2y-19	*Bacteroidetes*	FJ444670.1	100%
27	Uncultured Bacteroidetes bacterium clone Skagenf68	*Bacteroidetes*	DQ640698.1	100%
28	Pseudomonas sp. PcFRB037	γ-proteobacteria	AB548846.1	99%
29	Uncultured Bacteroidetes bacterium clone TH_a140	*Bacteroidetes*	EU373113.1	100%
30	Pseudomonas sp. S4.ACT.012	γ-proteobacteria	HM063879.1	100%
31	Uncultured Flavobacterium sp. clone U000130085	*Bacteroidetes*	FJ037438.1	89%
32	Acinetobacter sp. YF14	γ-proteobacteria	HQ684847.1	93%
33	Oxalobacteraceae bacterium enrichment culture clone MWE_C40	β-proteobacteria	FJ391493.1	97%
34	Trichococcus sp. 93	*Firmicutes*	EU325618.1	98%
35	Uncultured Chlorobium sp. clone 4.14	*Chlorobi*	GQ183422.1	99%
36	–	–	–	–
37	Uncultured gamma proteobacterium isolate DGGE gel band 13	*Proteobacteria*	EF127787.1	95%
38	Uncultured beta proteobacterium clone bulk33	β-proteobacteria	GU473062.1	93%
39	Uncultured beta proteobacterium clone TDNP_USbc97_13_7_13	β-proteobacteria	FJ516892.1	90%
40	Trichococcus sp. 93	*Firmicutes*	EU325618.1	100%
41	–	–	–	–
42	Leptothrix sp. OTSz_A003 partial 16SrRNA gene, strain OTSz_A003	β-proteobacteria	FM886835.1	98%
43	Uncultured Clostridiales bacterium clone 11-25; Uncultured Firmicutes bacterium gene, isolate: DGGE band: 75j	*Firmicutes*	GQ355062.1 AB541469.1	100%