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SHORT RESEARCH AND DISCUSSION ARTICLE

Influence of substrate type on microbial community structure in vertical-flow constructed wetland treating polluted river water

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Abstract Microorganisms attached on the surfaces of substrate materials in constructed wetland play crucial roles in the removal of organic and inorganic pollutants. However, the impact of substrate material on wetland microbial community structure remains unclear. Moreover, little is known about microbial community in constructed wetland purifying polluted surface water. In this study, Illumina high-throughput sequencing was applied to profile the spatial variation of microbial communities in three pilot-scale surface water constructed wetlands with different substrate materials (sand, zeolite, and gravel). Bacterial community diversity and structure showed remarkable spatial variation in both sand and zeolite wetland systems, but changed slightly in gravel wetland system. Bacterial community was found to be significantly influenced by wetland substrate type. A number of bacterial groups were detected in wetland systems, including Proteobacteria, Chloroflexi, Bacteroidetes, Acidobacteria, Cyanobacteria, Nitrospirae, Planctomycetes, Actinobacteria, Firmicutes, Chlorobi, Spirochaetae, Gemmatimonadetes, Deferribacteres, OP8, WS3, TA06, and OP3, while Proteobacteria (accounting for 29.1–62.3 %), mainly composed of Alpha-, Beta-, Gamma-, and Deltaproteobacteria, showed the dominance and might contribute to the effective reduction of organic pollutants. In addition, *Nitrospira*-like microorganisms were abundant in surface water constructed wetlands.

Keywords Constructed wetland · High-throughput sequencing · Microbial community · River water · Substrate

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Introduction

Due to its advantages of low cost, easy maintenance, and environmental friendliness, constructed wetland (CW) has been widely used in treatment of industrial, agricultural, and municipal wastewater (Chang et al. 2015; Ji et al. 2012; Mulling et al. 2014; Xiong et al. 2015; Zhi and Ji 2014) and polluted surface water (Dzakpasu et al. 2015; Tu et al. 2014). Biofilm microbial community, attached to the surfaces of substrate materials (or filter media) in CW, plays crucial roles in the reduction of degradable organic pollutants and transformation of inorganic pollutants including nitrogen, sulfate, and phosphate (Iasur-Kruh et al. 2010; Liu et al. 2013; Ramond et al. 2012). In addition, microbial assemblage is sensitive to operational and environmental conditions, and its structure is a good indicator for the status of CW ecosystem (Chang et al. 2015). Previous studies using traditional molecular biology approaches [e.g., denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), and clone library analysis] have greatly aided in our understanding of microbial community structure in CW ecosystem (Arroyo et al. 2013; Elsayed et al. 2014; Iasur-Kruh et al. 2010; Morato et al. 2014). A variety of factors have also been found to influence the structure of CW microbial community, such as layer depth (Bouali et al. 2014; Iasur-Kruh

et al. 2010), operational time (Bouali et al. 2014), plant type (Menon et al. 2013), plant species richness (Liu et al. 2013), plant development (Zhao et al. 2012), wastewater quality characteristics (Chang et al. 2015; Zhao et al. 2012), flow (Arroyo et al. 2013), and water depth (Morato et al. 2014). These previous studies mainly focused on CW systems treating industrial and municipal wastewater, yet microbial community in CW systems purifying polluted surface water has received little attention. Moreover, the impact of substrate material on CW microbial community structure remains unclear.

High-throughput sequencing technology is a highly efficient tool for identifying the profile of complicated microbial community (Liao et al. 2015; Wang et al. 2015a, b; Yang et al. 2015), and can provide a new opportunity to resolve the microbial assemblage of CW biofilm and systematically investigate its links with operational and environmental conditions (Ansola et al. 2014; Arroyo et al. 2015; Zhong et al. 2015). Due to its relatively low costs and great throughput, Illumina sequencing has been applied to profile microbial communities in various natural and man-made ecosystems (Kim et al. 2014; Shi et al. 2014; Sun et al. 2015; Wu et al. 2015; Yang et al. 2014; Zhang et al. 2015). However, information on high-throughput sequencing of microbial assemblage in CW treating surface water is still very limited (Ligi et al. 2014). Therefore, the main objective of the current study was to use Illumina high-throughput sequencing to investigate the impact of substrate type on the structure of microbial community in CW treating surface water.

Materials and methods

Wetland description

Three vertical-flow pilot-scale wetland systems (length 1.2 m, width 1 m, height 1 m) were constructed to treat the water of a heavily polluted river located in Guangdong Province (southern China). Each wetland system was filled with coarse gravel in the bottom layer (diameter 70–120 mm, height 20 cm). Wetlands WA, WB, and WC were filled with an 80-cm upper layer of sand (1–2 mm), natural zeolite (5–8 mm), and fine gravel (6–12 mm), respectively. The top of each wetland system was planted with *Pennisetum purpureum* Schum. at a density of nearly 30 plants/m^2. The retention time of river water in each wetland was set to 5 days. Before the beginning of experiments, these three wetland systems had been under continuous operation for nearly 1 year. During this period, the dissolved organic carbon (DOC) and ammonia nitrogen for the influents of each wetland system were 8–12 and 2–4 mg/L, respectively. The average DOC removal rates of wetlands WA, WB, and WC were 60, 79, and 49 %, respectively, while the average ammonia removal rate of each wetland system was above 95 %.

Molecular analyses

In the current study, substrate particle samples WA1–WA4, WB1–WB4, and WC1–WC4 were collected from 0.2, 0.4, 0.6, and 0.8 m below the surface of wetlands WA, WB, and WC, respectively. Total genomic DNA of each sample was extracted using Powersoil DNA extraction kit (Mobio Laboratories) according to the protocol recommended by the manufacturer. PCR amplicon libraries were constructed for Illumina MiSeq sequencing with the primer set 515F (5′-GTGCCAGCMGCCGCGG-3′)/R907 (5′-CCGTCAATTCMTTTRAGTTT-3′) that targets V4–V5 hypervariable regions of bacterial 16S rRNA genes (Wang et al. 2015b). The reads from raw DNA fragments were merged using FLASH and quality filtering of sequences was carried out according to the literature (Caporaso et al. 2011). Chimeric reads were checked and filtered out using UCHIME (Edgar et al. 2011), and the high-quality sequences were clustered into the operational taxonomic units (OTUs) by setting a 0.03 distance. Bacterial community richness (Chao 1 estimator) and diversity (Shannon index) were obtained using the UPARSE pipeline (Edgar 2013). The Classifier program of the RDP-II was used to assign taxonomic identity of the representative sequence from each OTU (Wang et al. 2007). The OTU-based beta diversity was calculated using UniFrac analysis to compare similarity among wetland microbial communities. Weighted UniFrac using Quantitative Insights into Microbial Ecology (QIIME) program was applied for weighted pair group method with arithmetic mean (WPGMA) clustering. The reads obtained from Illumina high-throughput analysis in this study were deposited in the NCBI short-read archive under accession number SRP059159.

Results and discussion

Bacterial community diversity

In this study, a total of 367,800 valid reads for 12 wetland samples were retrieved from Illumina MiSeq sequencing platform. Each library contained 11,466 to 28,561 reads, normalized to 11,460 for comparison of bacterial community diversity. Good's coverage estimator indicated that 96–99 % of the OTUs were retrieved in all wetland samples, using a 0.03 distance (Table 1), suggesting that the OTU diversity has been well captured. The OTU number ranged from 1203 to 2144 for each wetland sample. The Chao 1 estimator showed that the bacterial community richness ranged from 1622 to 2629 taxa in the three CW systems used to treat polluted river water. The bacterial community richness illustrated a remarkable

Table 1 Community richness and diversity indices for CW samples

Sample	OTUs[a]	Chao 1 estimator[a]	Shannon index[a]	Good's coverage (%)[a]
WA1	1424	1804	5.42	98
WA2	1203	1622	5.74	97
WA3	1664	1758	6.21	99
WA4	1570	1943	6.09	98
WB1	1903	2257	6.47	98
WB2	1512	1987	5.51	98
WB3	1591	2154	5.93	96
WB4	1692	2203	5.95	96
WC1	1654	2106	6.55	96
WC2	1907	2406	6.61	96
WC3	1992	2378	6.65	97
WC4	2144	2629	6.76	97

[a] Tags are normalized to 11,460

spatial variation in each CW system. The samples from wetland A showed lower richness than those from wetlands B and C. Moreover, the value of Shannon's diversity index of bacterial communities in wetland samples fell between 5.42 and 6.76. A remarkable spatial variation of Shannon's diversity was found in wetlands A and B, while Shannon's diversity only slightly increased with increasing wetland layer depth. The samples from wetland C showed higher Shannon's diversity than those from wetlands A and B.

In a large-scale CW system purifying polluted river water, DGGE analysis indicated that Shannon's diversity of wetland sediment bacterial community decreased from inlet zone (3.471) to outlet zone (2.566) (Zhi et al. 2015). In this study, high-throughput sequencing was used to profile the microbial communities in surface water CW systems. The observed Shannon's diversity (5.42–6.76) in this study was much higher than that reported in a previous study using DGGE analysis (Zhi et al. 2015). In addition, a remarkable spatial variation of bacterial community diversity was observed in both sand and zeolite CW systems, instead of gravel CW system. The gravel CW system harbored higher bacterial community diversity than the sand and zeolite CW systems. These results suggested the strong impact of substrate type on bacterial community diversity in surface water CW system. In contrast, Huang et al. (2013) reported a weak impact of substrate type on bacterial community diversity in CW treating piggery wastewater.

Bacterial community structure

In this study, only a small proportion of sequences retrieved from the three surface water CW systems could not be affiliated with known bacterial phylum or candidate division (accounting for 0.17–6.35 %) (Fig. 1). A total of 13 bacterial phyla and four candidate divisions were frequently identified among 12 wetland samples, including Proteobacteria, Chloroflexi, Bacteroidetes, Acidobacteria, Cyanobacteria, Nitrospirae, Planctomycetes, Actinobacteria, Firmicutes,

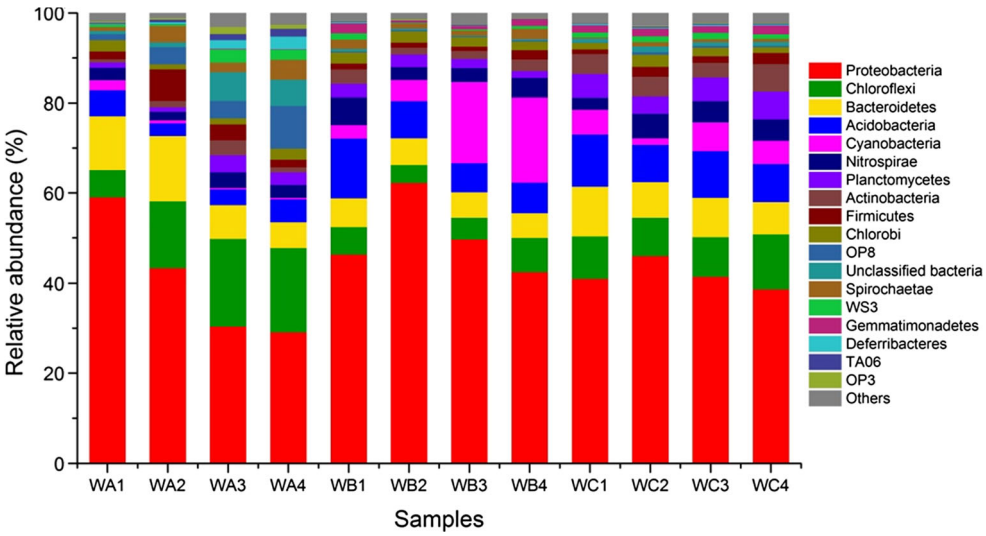

Fig. 1 Comparison of the quantitative contribution of the sequences affiliated with different phyla to the total number of sequences from CW samples. The rare species with relative abundance of less than 0.5 % in each sample are included as others

Chlorobi, Spirochaetae, Gemmatimonadetes, Deferribacteres, OP8, WS3, TA06, and OP3. Among these bacterial phyla, Proteobacteria (accounting for 29.1–62.3 %) was the largest bacterial group in all the wetland samples. Phylum Chloroflexi (4–19.4 %) was the second largest bacterial group in samples WA2, WA3, WA4, WC2, and WC4, and was also dominant in other wetland samples. The relative abundance of Bacteroidetes and Acidobacteria in the wetland samples was 5.5–14.5 % and 2.8–13.3 %, respectively. Cyanobacteria showed high proportion in samples WB3 (18 %) and WB4 (19 %), but became much less abundant in other wetland samples (0.4–6.4 %). The organisms belonging to phyla Nitrospirae, Planctomycetes, Actinobacteria, Firmicutes, and Chlorobi were also abundant in all the wetland samples (>1 %). In addition, the relative abundance of these major bacterial groups usually illustrated a remarkable spatial variation in each CW system.

Figure 2 illustrates the composition of proteobacterial community in each wetland sample. Epsilonproteobacterial organisms only showed very low proportion in wetland samples (below 0.3 %). The wetland proteobacterial communities were mainly composed of Alpha-, Beta-, Gamma-, and Deltaproteobacteria. The samples from wetland WA (1–7.7 %) had much lower proportion of alphaproteobacterial microorganisms than those from the other two wetlands (12–28.2 %). Except for sample WA1 (35.1 %), the samples from wetland WA (10.7–18.6 %) also showed lower proportion of betaproteobacterial species than those from the other two wetlands (26.3–38.6 %). Moreover, betaproteobacterial proportion was generally much higher than alphaproteobacterial proportion in the studied wetland samples. Gammaproteobacterial proportion (10.2–45.5 %) was also usually lower than betaproteobacterial proportion in wetland samples. The samples from wetland WA (32.2–77.6 %) had much higher deltaproteobacterial proportion than those from the other two wetlands (13.1–31.4 %). In addition, the relative abundance of these major proteobacterial classes also illustrated a large spatial variation in each CW system.

The result of WPGMA clustering based on OTU level illustrated that all of the studied wetland samples fell into three distinctive clusters (Fig. 3). Except for sample WB1, samples from the same wetland were grouped together. This suggested that substrate type could have a profound impact on microbial community structure. However, for the four samples in each wetland, the ones from 0.6 and 0.8 m below the wetland surface were more closely clustered, which showed the strong impact of layer depth on microbial community structure.

Microorganisms from Proteobacteria might be involved in the biodegradation or biotransformation of numerous organic compounds in natural or manmade ecosystems (Cheng et al. 2014; Liao et al. 2013a, 2015; Liu et al. 2014a; Zhang et al. 2014). The dominance of Proteobacteria has been found in natural wetlands (Ansola et al. 2014; Liu et al. 2014b), wastewater CW systems (Ansola et al. 2014; Bouali et al. 2014; Huang et al. 2013), and biofilters treating surface water (Feng et al. 2013a; Liao et al. 2013b). Moreover, a previous study using DGGE analysis also suggested the dominance of proteobacterial organisms in a large-scale CW system

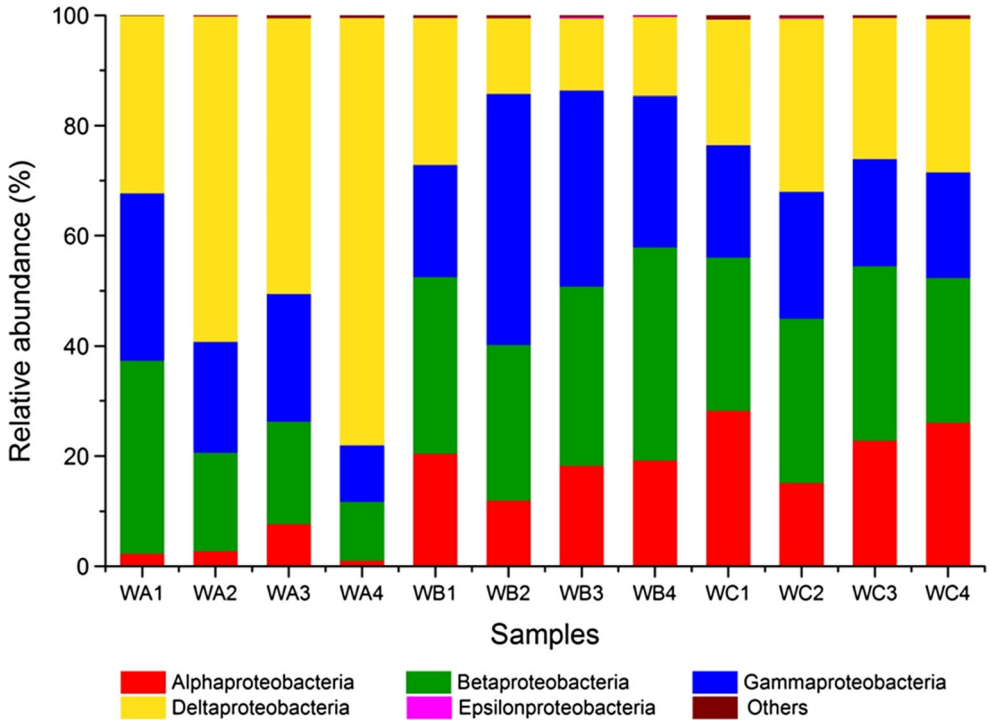

Fig. 2 Comparison of the quantitative contribution of the sequences affiliated with different proteobacterial classes to the total number of proteobacterial sequences from CW samples. Proteobacterial sequences not related to any known proteobacterial class are included as others

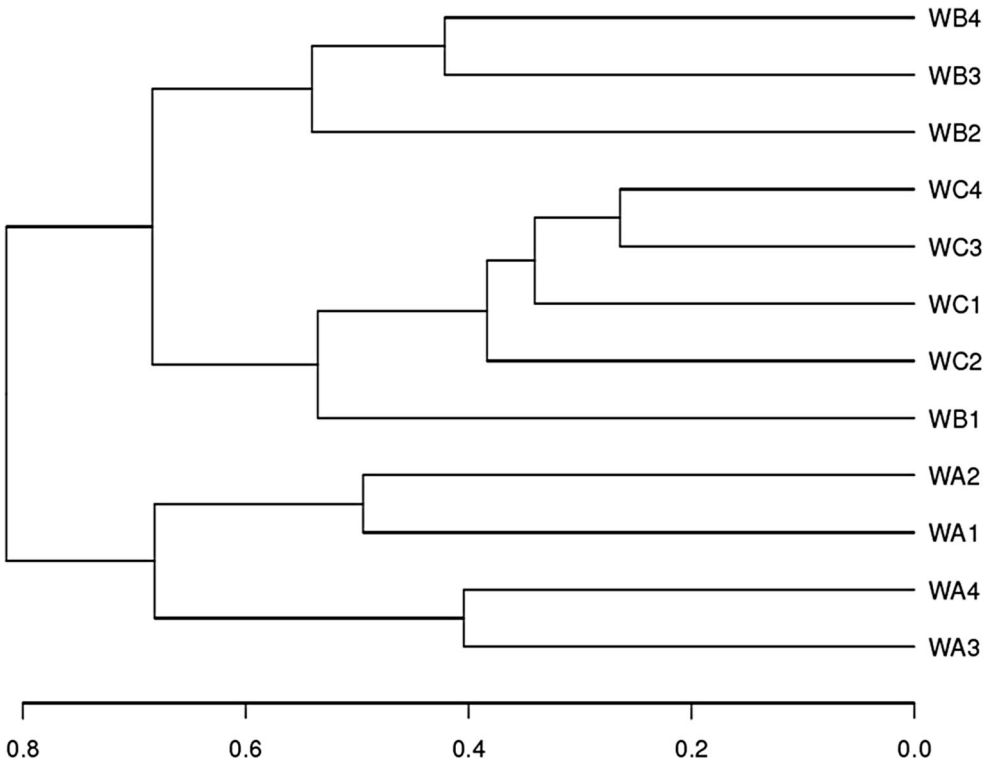

Fig. 3 WPGMA clustering of CW samples

purifying polluted river water (Zhi et al. 2015). To date, the influential factors governing the structure of wetland proteobacterial community remain unclear. The spatial variation of proteobacterial proportion in surface water CW has never been addressed. In this study, Illumina MiSeq sequencing analysis also showed the dominance of Proteobacteria in surface water CW. However, a remarkable spatial variation of proteobacterial proportion was observed in the sand and zeolite CW systems, while the gravel CW system showed a relatively slight change. In addition, the proportion of major proteobacterial classes also experienced a more profound spatial shift in the sand and zeolite CW systems, compared with the gravel CW system. These results suggested the strong impact of substrate type on proteobacterial community structure in surface water CW system.

So far, the impact of substrate material on CW microbial community structure remains in debate. Silyn-Roberts and Lewis (2004) revealed a very slight difference of CW microbial population structure between biofilms on slag and on greywacke, while Huang et al. (2013) showed the significant impact of substrate type on CW microbial community structure. In this study, the result of WPGMA clustering further confirmed the profound impact of substrate type on the structure of total bacterial community in surface water CW system.

Bacterial genera

There were a total of 33 frequently detected genera in the 12 studied wetland samples. The difference of bacterial community structure among these wetland samples was also evident at genus level (Table 2). For an example, most of the frequently detected genera only showed dominance (with relative abundance of 1 %) in a sole wetland sample. Microorganisms from genera *Arenimonas* and *Thermomonas* showed relatively high proportion in samples WB2, WB3, and WB4 (>1 %). Moreover, *Planktothrix* species dominated in sample WB4 (11.55 %), but became less abundant in other wetland samples. Although *Nitrospira* was dominant in each wetland sample, its proportion illustrated a large shift (1.89–6.12 %).

Microorganisms from proteobacterial genera *Burkholderia*, *Pseudoxanthomonas*, *Smithella*, *Syntrophobacter*, *Syntrophorhabdus*, and *Syntrophus* are able to aerobically or anaerobically degrade various organic compounds (Arora et al. 2014; Chen et al. 2005, 2013; Gan et al. 2014; Liu et al. 1999; Mouttaki et al. 2008; Nopcharoenkul et al. 2013; Patel et al. 2012; Qiu et al. 2008). Members of genus *Flavobacterium* (Bacteroidetes) have been linked to biodegradation of a variety of organic pollutants (Nedashkovskaya et al. 2014; Sack et al. 2011; Sun et al. 2011). In addition, microorganisms within genus *Bacillus* (Firmicutes) can biodegrade a number of organic compounds (Chebbi et al. 2014; Nakkabi et al. 2015; Patowary et al. 2015; Reddy et al. 2014; Xiao et al. 2015; Zhao et al. 2014). Therefore, the dominance of microorganisms from a variety of bacterial genera might play important roles in the removal of organic compounds in the three CV systems and thus contribute to the effective reduction of DOC in polluted river water. Moreover, *Nitrospira* species (Nitrospirae) are known for their

Table 2 Comparison of percentage of the sequences affiliated with the frequently identified genera to the total number of sequences from wetland samples

Taxon	WA1	WA2	WA3	WA4	WB1	WB2	WB3	WB4	WC1	WC2	WC3	WC4
Alphaproteobacteria												
Parvibaculum	–[a]	–	–	–	2.12	–	–	–	–	–	–	–
Roseomonas	–	–	–	–	–	1.07	–	–	–	–	–	–
Betaproteobacteria												
Burkholderia	–	–	1.27	–	–	–	–	–	–	–	–	–
Rivibacter	–	–	–	–	–	1.12	–	–	–	–	–	–
Thiobacillus	–	–	–	–	1.43	0.99	0.59	1.79	–	–	–	–
Thiomonas	–	–	–	–	–	–	–	1	–	–	–	–
Gammaproteobacteria												
Arenimonas	–	–	–	–	–	2.25	1.3	2.1	0.88	–	–	–
Pseudoxanthomonas	–	–	1.21	–	–	–	–	0.58	–	–	–	–
Stenotrophomonas	–	–	1.01	–	–	–	–	–	–	–	–	–
Thermomonas	–	–	–	–	–	4.13	4.12	1.94	–	–	–	–
Thioalkalispira	–	–	–	–	1.03	–	–	–	–	–	–	–
Deltaproteobacteria												
Anaeromyxobacter	1.03	0.73	0.72	–	0.78	–	–	–	–	0.54	–	–
Smithella	–	4.34	–	–	–	–	–	–	–	–	–	–
Syntrophobacter	–	1.05	–	–	–	–	–	–	–	–	–	–
Syntrophorhabdus	1.66	2.65	2.36	1.50	1	0.58	–	–	–	0.98	0.82	0.6
Syntrophus	–	1.73	–	–	–	–	–	–	–	–	–	–
Acidobacteria												
Blastocatella	–	–	–	–	–	1.21	0.95	–	0.51	–	–	–
Candidatus_Koribacter	–	–	–	–	1.31	–	–	0.5	–	–	–	–
Geothrix	–	–	–	–	1.27	0.58	–	0.83	–	–	–	–
Actinobacteria												
Gaiella	–	–	–	–	–	–	–	–	0.87	1.14	0.76	1.41
Bacteroidetes												
Chitinophaga	0.58	–	–	–	–	1.03	–	–	–	–	–	–
Dysgonomonas	–	–	1.96	–	–	–	–	–	–	–	–	–
Flavobacterium	–	–	–	–	0.94	–	–	–	–	–	–	–
Roseiflexus	–	–	–	–	–	–	–	–	–	–	0.98	0.81
Chloroflexi												
Roseiflexus	–	–	–	–	–	–	–	–	0.96	–	0.63	0.77
Cyanobacteria												
Chroococcidiopsis	–	–	–	–	–	–	3.47	–	–	–	–	–
Microcoleus	–	–	–	–	1.3	–	–	–	–	–	–	0.69
Planktothrix	–	–	–	–	–	0.71	4.57	11.55	–	–	–	–
Deferribacteres												
Calditthrix	–	–	1.69	2.24	–	–	–	–	–	–	–	–
Firmicutes												
Bacillus	–	–	2.09	–	–	–	–	0.9	–	–	–	–
Gelria	–	3.02	–	–	–	–	–	–	–	–	–	–
Nitrospirae												
Nitrospira	2.75	1.89	3.47	2.83	6.12	2.88	3.04	4.3	2.64	5.43	4.71	4.79
Spirochaetae												
Spirochaeta	–	2.68	1.91	4.01	1.82	0.81	0.89	1.79	–	0.81	0.62	0.56

[a] Less than 0.5 %

role in nitrite oxidation (Feng et al. 2013b; Liao et al. 2013b). In this study, *Nitrospira*-like organisms showed high relative abundance in each wetland sample, suggesting the presence of strong nitrifying activity in the three CV systems. This might indirectly explain the effective removal of ammonia in polluted river water.

Conclusions

Illumina high-throughput sequencing indicated that bacterial community diversity and structure varied remarkably in sand and zeolite CW systems treating polluted river water, but slightly in gravel CW system. Substrate type could have a strong impact on bacterial community in surface water CW system. Proteobacteria dominated in all the three CW systems. Microorganisms from a variety of bacterial genera might contribute to the effective reduction of DOC. Moreover, *Nitrospira* illustrated high relative abundance in each CW system.

Ethical statement No conflict of interest exists in this manuscript. The work has not been published previously and not under consideration for publication elsewhere.

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