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Effect of reclaimed water effluent on bacterial community structure in the *Typha angustifolia* L. rhizosphere soil of urbanized riverside wetland, China

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ABSTRACT

In order to evaluate the impact of reclaimed water on the ecology of bacterial communities in the *Typha angustifolia* L. rhizosphere soil, bacterial community structure was investigated using a combination of terminal restriction fragment length polymorphism and 16S rRNA gene clone library. The results revealed significant spatial variation of bacterial communities along the river from upstream and downstream. For example, a higher relative abundance of γ-Proteobacteria, Firmicutes, Chloroflexi and a lower proportion of β-Proteobacteria and ε-Proteobacteria was detected at the downstream site compared to the upstream site. Additionally, with an increase of the reclaimed water interference intensity, the rhizosphere bacterial community showed a decrease in taxon richness, evenness and diversity. The relative abundance of bacteria closely related to the resistant of heavy-metal was markedly increased, while the bacteria related for carbon/nitrogen/phosphorus/sulfur cycling wasn't strikingly changed. Besides that, the pathogenic bacteria markedly increased in the downstream rhizosphere soil since reclaimed water supplement, while the possible plant growth-promoting rhizobacteria obviously reduced in the downstream sediment. Together these data suggest cause and effect between reclaimed water input into the wetland, shift in bacterial communities through habitat change, and alteration of capacity for biogeochemical cycling of contaminants.

Introduction

Centralized reclaimed water treatment plants (RWTPs) are one of the most common systems for the treatment of domestic wastewater in highly urbanized areas with high population densities in Beijing, China. Therefore, RWTPs effluent represents a significant component of the supplement water of river ecosystems in Beijing. Currently, the use of reclaimed water in Beijing amounts to 2.3×10^8 m^3, 1.5×10^8 m^3 (65.2%) of which are used by lakes and rivers. However, numerous studies have documented that reclaimed water is rich in nitrogen, phosphorus and other nutrients; also, as a result of the slow stream flow of urban rivers, their long update cycle, and the single body of water ecosystem structure, the water column potentially has a higher risk of eutrophication and temporary oxygen deficits (Zhou et al.,

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2006; Meng et al., 2011). In addition, domestic wastewater is normally anthropogenic, such as municipal and hospital producing wastewater, which contains residual Pharmaceutical and Personal Care Products (PPCPs), and biologically active metabolites. These contaminants can mix into the natural water cycle through reclaimed water supplement (Yu et al., 2011; Tong and Wei, 2012; Knapp et al., 2012). Reclaimed water was one of the chlorine sources, which could produce organic pollutant, substitution reactions, disinfection byproducts, chloroform, carbon tetrachloride, *etc.* Thus, reclaimed water is an important source of water transmitted antibiotics and cross resistance genes from disinfection byproduct, which result in higher potential risk to human health (Dong et al., 2010).

The quality of reclaimed water affects the functions and biological processes of receiving river directly (Wassen et al., 1992), particularly when it enters waterways during periods of low natural flow. The urban artificial wetland, considered as a natural alternative to technical methods of wastewater treatment, which can effectively remove nitrogen, phosphorus, heavy metals oxide, various organic substances and pathogens, and reduce BOD and TSS content in water (Thurston et al., 2001; Zhao et al., 2007). Thus, urban artificial wetlands have gradually been widely used in improving water quality in urban lake and river landscapes (Cui et al., 2011). Many studies had pointed out that the interaction matrix, aquatic plants and microorganisms in the wetland system was the main mechanisms of effluent purification (Toyama et al., 2009). Among them, microorganisms play an imperative role in the process of purification, especially the plant rhizosphere bacteria. Wetland plants had a special ability called the "rhizosphere effect," which allows microbes in the plant rhizosphere to enhance the carrying capacity of constructed wetlands (Xiang et al., 2004). The rhizosphere bacterial communities propagate at high speed, with high abundances and strong metabolizing abilities (Feng et al., 2012), which play a very important role in the processes of removing, fixing and conversion of nitrogen, phosphorus and other organic/inorganic matters, including heavy metal removal, *etc.* (Jing and Yang, 2004; Zhang et al., 2007; Nicomrat et al., 2008; Li, 2012). Therefore, rhizosphere bacterial communities are the main force involved in the degradation of pollutants and play an important role in maintaining the ecological balance and achieving ecological purification of wetland systems. Characteristics of microbial biomass, activity and community composition in constructed wetlands can be directly affected by hydraulic conditions, wastewater properties, including substrate and nutrient quality and availability, filter material or soil type, plants, and other environmental factors (Truu et al., 2009). Therefore, wetland plant rhizosphere microbial characteristics can sensitively reflect the status of plant and water quality, which are considered ideal indicators of aquatic ecosystems and have been extensively used to assess the degree of toxicity imposed by various pollutants. To the best of our knowledge, most studies have focused on the impacts of wastewater treatment plants (WWTPs) effluent on bacterial communities in the water column and sediment of receiving river (Wakelin et al., 2008; Drury et al., 2013). But few studies have investigated wetland plant rhizosphere bacteria community under reclaimed water disturbance condition.

We aimed to characterize the effect of RWTPs effluent on the structure of bacterial communities in the rhizosphere bacterial community of *Typha angustifolia* L. in urbanized riverside wetland in our study. A combination of terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA library technique was used to investigate the diversity, abundance and function of bacterial community in *T. angustifolia* rhizosphere samples collected from near the reclaimed water outfall, 300 m upstream the RWTP outfall and 2000 m downstream the RWTP outfall. The two methods exhibit different properties in the analysis of community structure. T-RFLP which is widely applied to the fields of biodiversity analysis and comparison of microbial community has crucial theoretical meaning and practical applied value (Wang et al., 2012). However, it is not applicable to describe community composition because of some shortcomings. For example, phylogenetic identification is problematic because some terminal restriction fragments (T-RTs) can't match the corresponding species or genus of bacteria from database (Marsh, 1999). Conversely, the 16S rRNA clone library method is not suitable for analyses of community diversity since the limitation of conversion efficiency. However, for community composition analysis, the 16S rRNA clone library method, which has the highest resolution ability, appears to be suitable (Guo et al., 2015). Narrowleaf cattail (*T. angustifolia*) is one of the most common plants in constructed wetlands used for wastewater treatment. Earlier studies have shown that cattails' endophytic bacteria can assimilate nutrients (Whitacre, 2012), heavy metals (Demirezend and Aksoy, 2004) and phytoremediate eutrophic water bodies (Cristina et al., 2009) in the constructed wetland or semi-natural treatment wetland. But no study has investigated the effect of reclaimed water on the rhizosphere bacterial community of *T. angustifolia* in urbanized riverside wetland. Date from this study can provide better understanding of the interactions between reclaimed water variables and complex bacterial communities in wetland systems, as well as useful information of indigenous populations with potential application to reclaimed water purification. It also can provide a scientific reference to maintain the ecological balance and construct the high-efficiency wetland purification system.

1. Materials and methods

1.1. Description of study area

The Beijing Yongding River artificial wetland was 5.24-km-long, which geographically begins in the town of Sanjiadian, and extends to Mayu Village, located in Mentougou District. This artificial wetland mainly uses reclaimed water as a supplemental water source to sustain the water inflow in the wetland culverts. The mean water depth in the study area is 1.5–2.5 m. Water storage capacity is 1.6×10^6 m^3. The average annual rainfall of whole watershed is about 556–560 mm, and rainfall mainly concentrates in 6–9 months. To form the wetland landscape, a dam of Sanjiadian reservoir was set up in the upstream of the drainage outlet. Two major reclaimed water outfalls (Zhongmensi ditch outfall and Gaojing canal outfall) are located in approximately 4000 m downstream of the reservoir

dam, and amount of annual supplied reclaimed water is about 2×10^6 m^3. Supplementary reclaimed water of wetland was mainly discharged from Mentougou Wastewater Treatment Plant, where the A^2/O + MBR (membrane bioreactor) treatment process were adopted to treat urban sewage and partial industrial wastewater surrounding the plant. Aquatic plants in artificial wetland play an important role in reclaimed water purification. The most abundant aquatic plant in artificial wetland is cattails (*T. angustifolia*), and the vegetation coverage in artificial wetland reaches 70%–90%.

1.2. Sample collection and analysis

Typha rhizosphere samples were taken from four locations along the artificial wetland (Fig. 1) during October 2012. According to the distribution of Typha in the wetland, the locations were 300 m upstream of the RWTP outfall (inferred as an undisturbed reference site with respect to the outfall), immediately above and below the outfall, and then downstream at 2000 m below the outfall. At each location, five replicate Typha rhizosphere soil samples were taken and the sampling points were evenly distributed from the center to both sides of the river, and then evenly blended into one typical sample of Typha rhizosphere. Almost all sampling sites had a depth of 30 cm. The Typha had a height of about 2.5 m. The samples were immediately mixed and transported

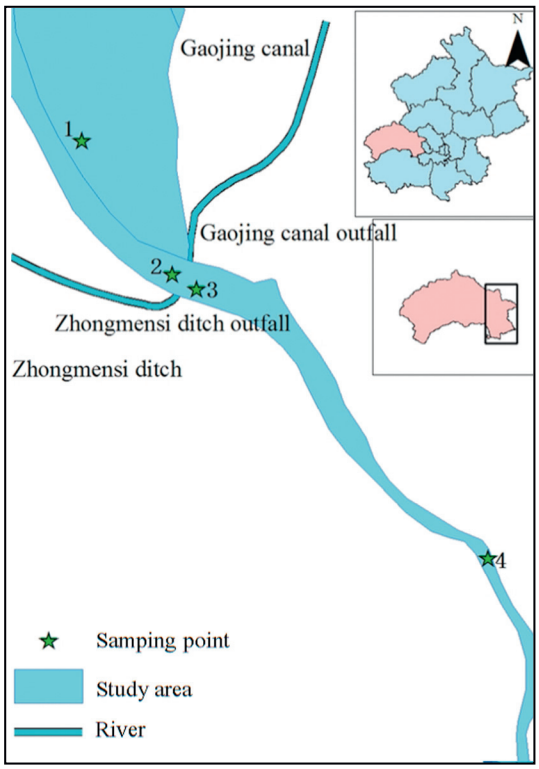

Fig. 1 – Map of artificial wetland in Yongding River, the RWTP, and the sampling locations. *Typha* rhizosphere samples were taken from four locations along the artificial wetland, location 1 is "undisturbed" with respect to the location of the RWTP outfall, locations 2 and 3 are immediately above and below the outfall, location 4 is 2000 m downstream of the outfall.

to the laboratory. After shaking down loose attachments from the Typha roots, about 0.5 g of the roots were placed in sterile flasks, and 200 mL sterile ddH_2O was added. The samples were stirred for 15 min in 300 r/min. Subsequently, the plant roots were removed, and then 0.22 μm membrane filter was used to remove moisture, and the plant rhizosphere microbial sample was kept on the membrane. Samples on membrane were divided into two parts. One part of sample was used to analyze bacterial community. Another part of sample was carried out conventional physicochemical index analysis. The physicochemical index analytical methods were as follows: determination of total nitrogen (TN) used the Kjeldahl Method (GB/T11894-1989), the total phosphorus (TP) was detected by alkali fusion-Mo-Sb Anti spectrophotometric method (HJ632-2011), the total organic carbon (TOC) was measured by Potassium Dichromate Oxidation Spectrophotometric Method (HJ615/2011), the ammonium nitrogen was measured by 2 mol/L KCl leaching indophenol blue colorimetric method, the oxidation–reduction potential of soil sample (ORPs) was measured directly by the oxidation–reduction potential instrument. Furthermore, pH, *T* (temperature), ORPw (oxidation–reduction potential water sample), Sal (salinity), TDS (total dissolved solid), DO (dissolved oxygen), Chl-*a* (chlorophyll *a*) were detected by a water quality meter (Hydrolab Datasonde5, 5×, the United States) through on-site measurement.

1.3. DNA extraction and PCR amplification

DNA was extracted from 0.5 g rhizosphere soil using PowerSoil™ DNA Isolation Kit 12888-50 (MO BIO, Inc., Solana Beach, CA), following the manufacturer's instructions. The quality and quantity of extracted DNA were verified by agarose gel electrophoresis.

Polymerase chain reaction (PCR) was performed with a pair of primers 27f (5′- (6-FAM)-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Shanghai Sangon Biotech Co. Ltd., China) to amplify the 16S rRNA of the cattails rhizosphere bacteria. The 25 μL PCR mixture contained 2 μL of genomic DNA, 12.5 μL 2× Taq reaction buffer, 1 μL of primer 27f (10 μmol/L) and 1 μL of primer 1492r (10 μmol/L) and 8.5 μL ddH_2O. PCR cycling parameters consisted of an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles of 95°C for 50 sec, 55°C for 50 sec, 72°C for 1 min and a final elongation at 72°C for 7 min. After fluorescent PCR amplification products were identified using 0.8% (*m*/*V*) agarose gel electrophoresis, then wrapped with tinfoil and stored at 4°C.

1.4. T-RFLP analysis and phylogenetic assignment

All T-RFLP analyses were carried out as previously described (Dunbar et al., 2001; Blackwood et al., 2003; LaMontagne et al., 2003). Briefly, all fluorescently labeled PCR amplification products were digested with *Msp*I, *Hha*I and *Rsa*I enzymes (Takara Co., Japan) at 37°C for 4 hr. Each 10 μL digestion reaction contained 5 μL of PCR products, 1 μL 10× Taq reaction buffer, ddH_2O 3.5 μL added, 0.5 μL of *Msp*I, *Hha*I and *Rsa*I enzymes in separate reactions at 37°C for 4 hr. And then incubate for 15 min under the condition at 65°C to make enzyme inactivation. After that, Genomic Scanning of

enzyme-digested products was carried out by Tiangen Biotech (Beijing) Co., Ltd., China to obtain T-RFLP profiles. Fragments larger than 500 or smaller than 50 bp were deleted from analysis. Relative abundance of each terminal restriction fragments (T-RFs) was calculated and the T-RFs with relative area percents less than 1% were also deleted from further analysis.

The web-based, T-RFLP Phylogenetic Assignment Tool (PAT), was used for designation of names to T-RFs. The reference database used was the default database for PAT, generated using the Microbial Community Analysis query function found at the MiCA website (http://mica.ibest.uidaho.edu/). Only phylogenetic assignments that have matched T-RF lengths in all two restriction enzymes were used, and the rest was discarded.

The cluster analysis was performed based on the T-RFLP profiles using the PRIMER 5 software. Shannon diversity index (H') was determined using Eq. (1):

$$H' = -\sum(p_i)(\ln p_i) \quad (1)$$

while evenness (J') was calculated as Eq. (2):

$$J' = H'/\ln S \quad (2)$$

and Simpson's diversity index ($1/D$) as Eq. (3):

$$(1/D) = 1/\sum p_i^2 \quad (3)$$

where, p_i is the relative abundance of T-RFs, S is the number of T-RFs, and D is for Simpson's dominance index, which is inversely proportional to diversity.

1.5. 16S rRNA gene clone library and phylogenetic analysis

Bacterial 16S rRNA gene clone libraries were constructed from the Typha rhizosphere samples of upstream and downstream of the RWTP outfall. The PCR condition and primers were the same as those used for bacterial T-RFLP analysis, except that the forward primer was not labeled with 6-FAM. Concentration detection of the purified PCR amplification products was done by the Biophotometer in conjugation experiments. The purified PCR products were ligated into the pGEM-T Easy vector (Promega Co., the United States). Subsequently, *Escherichia coli* Trans1-T1 competent cells (TransGen Biotech Co., China) were transformed with the ligation products and spread onto LB agar plates with ampicillin (100 mg/L), X-gal and IPTG for standard blue and white screening (Sambrook and Russell, 2001). Randomly selected colonies were checked directly by PCR amplification with primers SP6 (ATTTAGGTGACACTATAGAA) and T7 (TAATACGACTCACTATAGGG) for the vector (Takara Co., Japan). And a total of 420 positive clones from the upstream library and 320 positive clones from the downstream library were sequenced using an ABI PRISM 3730 automatic sequencer (Shanghai Sangon Co. Ltd., China).

The obtained sequence data were tested by the Chimera-Check program (www.cardiff.ac.uk/biosi/research/biosoft) to remove artificial chimerical sequences. The remaining sequences within each library were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity using the furthest-neighbor algorithm in Mothur software (Schloss et al., 2009). The get.oturep command in Mothur was used to select representative sequences and they were used for all subsequent analyses (Schloss et al., 2009; Sullam et al., 2012). The taxonomic affiliation of each representative sequence was compared with the NCBI database using BLASTN or aligned by the identify analysis of EzTaxon server 2.1. Sequences with >97% similarity were assigned to the same species. The selected strains all had the highest sequence homology and were qualified published. MEGA 5 software was used for analysis. Then, Clustal W software was used to sort the samples according to maximum homology.

1.6. Coverage of clone libraries

To estimate the representation of the library, coverage of clone library (C, %) was calculated by the following equation based on the sequencing results:

$$C = (1–n/N) \times 100\% \quad (4)$$

where, n is the number of single clones, and N is the total number of clones in the clone library.

2. Results

2.1. Water quality and soil physicochemical properties in T. angustifolia rhizosphere sampling site

The detection results of water quality characteristics and physicochemical properties of *T. angustifolia* rhizosphere soil in sampling sites are shown in Table 1. For these detected physicochemical properties of the water and sediment, ANOVA (analysis of variance) and Tukey's HSD were performed to assess differences between different spatial points. ORPs, NH_4^+-N, pH, ORPw, Sal, TDS, DO and Chl-a were screened out by one-way ANOVA ($p < 0.05$), which means that those indicators were significantly affected by reclaimed water supplement and wetland purification. As shown in Table 1, the water quality indicators Sal, TDS and Chl-*a* were markedly higher in the pool below outfall than in the pool above outfall because of the reclaimed water supplement. ORPs increased slightly in the pool below outfall with DO decrease dramatically, maybe due to the disturbance of rhizosphere soil caused by reclaimed water supplement, while the significant reduction happened in the downstream. Besides that, the NH_4^+-N was also significantly increased in the near outfall, while it was significantly decreased in 2000 m below outfall. Obviously, the wetlands play an important role in removing NH_4^+-N, and the removal efficiency was 45.83% by concentration depletion. Compared to the other sampling sites, the sediment physicochemical indicators TN, TP, TOC were slightly higher in the upstream, this may have been due to the reduction in flow resulted in the deposition of organic matter and other material.

2.2. Diversity of rhizosphere bacterial community as determined by T-RFLP

Community diversities were estimated by number of ribotypes, evenness index, Shannon and Simpson's diversity index based on the T-RFLP profiles (Table 2). The chart in Table 2 shows that

Table 1 – Sampling site characteristics.

Sample	Parameter	300 m above outfall	Pool above outfall	Pool below outfall	2000 m below outfall
Soil sample	TN (g/kg)	1.33 ± 0.36 a *	1.02 ± 0.02 a	1.21 ± 0.08 a	1.07 ± 0.21 a
	TP (μg/mL)	0.78 ± 0.03 a	0.37 ± 0.01 a	0.7 ± 0.13 a	0.53 ± 0.21 a
	ORPs (mV)	−88.3 ± 1.47 ab	−52.85 ± 1.20 ab	−24.15 ± 2.33 a	−136.63 ± 4.68 b
	TOC (g/kg)	5.73 ± 0.61 a	1.77 ± 0.05 a	2.33 ± 0.29 a	2.76 ± 0.47 a
	NH_4^+-N (mg/kg)	3.20 ± 0.21 a	13.96 ± 0.06 ab	25.60 ± 2.28 b	8.45 ± 3.76 a
Water sample	pH	9.67 ± 0.58 a	10.62 ± 0.02 b	10.13 ± 0.01 ab	10.12 ± 0.00 ab
	Temp. (°C)	25.54 ± 0.92 a	25.88 ± 0.02a	26.34 ± 0.06 a	26.38 ± 0.00 a
	ORPw (mV)	308.83 ± 20.98 a	274.5 ± 0.71 b	281.58 ± 0.59 ab	282 ± 0.00 ab
	Sal (ng/L)	0.33 ± 0.01 a	0.28 ± 0.01 b	0.31 ± 0.00 a	0.31 ± 0.00 a
	TDS (g/L)	0.4 ± 0.00 a	0.3 ± 0.00 b	0.4 ± 0.00 a	0.4 ± 0.00 a
	DO (mg/L)	9.27 ± 0.32 a	6.26 ± 0.08 b	0.34 ± 0.00 c	0.34 ± 0.00 c
	Chl-*a* (μg/L)	24.85 ± 14.54 a	30.27 ± 0.00 a	141.77 ± 30.24 b	163.15 ± 0.00 b

ORPs/ORPw: oxidation–reduction potential; Sal: salinity; TDS: total dissolved solid.
* Values given are the means of three replicates ± standard deviation. Means followed by the same letter in the same line are not significantly different ($p < 0.05$).

RWTP effluent strikingly changed the rhizosphere bacterial communities structure characteristics, which led to bacterial community richness index, diversity index and evenness index decrease dramatically in the near outfall. However, they were markedly increased in the 2000 m below outfall relative to the near outfall. Briefly, the result shows that a decrease in bacterial richness, evenness and diversity with an increase of the reclaimed water interference intensity. According to the above analyzed result, we found that community diversities generated by the *Msp*I and *Hha*I enzymes digestion were slightly higher than that by the *Rsa*I enzyme. So, the following cluster analysis was performed based on the result of the *Msp*I and *Hha*I enzymes digestion. The cluster analysis results generated by the two restriction enzymes were extremely consistent (Fig. 2). There were significant differences in the bacterial community structure according to location. Results of the clustering analysis showed that two samples derived from the near outfall have a low degree of similarity. However, the similarity was higher relatively between the 2000 m below outfall sampling site and the 300 m above outfall sampling site, which means that the diversity of rhizosphere bacterial communities was markedly changed at the near outfall locations because of reclaimed water supplement and then restored gradually in the downstream.

2.3. Comparison of taxonomic composition of upstream and downstream rhizosphere soil samples based on T-RFLP profile

From the phylum diversity result, it was found that RWTP effluent did have a marked impact on the bacterial community diversity (Fig. 3). Overall, the bacterial communities in the upstream point contained 15 bacterial phyla. The predominant phyla belonged to γ-Proteobacteria, Actinobacteria, β-Proteobacteria and Bacteroidetes, with the remainder spread among Firmicutes, ε-Proteobacteria, Fusobacteria, and other minor groups. While the phylum number of bacterial community in the downstream point was 11, which decreased 60% than that of upstream point. In detail, the diversity of γ-Proteobacteria, β-Proteobacteria, ε-Proteobacteria, Actinobacteria, Bacterodetes, Firmicutes and Fusobacteria was decreased. However, the diversity of Planctomycetes, α-Proteobacteria, and δ-Proteobacteria was increased in the downstream. It is noteworthy that the bacterial community diversity was low extremely near the outfall, and only three matched bacteria were obtained from the database, including *Delftia* sp., *Flavobacterium* sp. and *Thermotoga* sp.

2.4. Comparison of taxonomic composition of upstream and downstream rhizosphere soil samples based on 16S rRNA gene clone library analysis

In order to further understand the differences of rhizosphere bacterial function and community structure between the upstream and downstream locations, *T. angustifolia* rhizosphere bacterial communities derived from the upstream and downstream were explored using a 16S rRNA library. A total of 420 positive clones were obtained from the upstream library. Among them, 28 bad sequences were deleted and the

Table 2 – Richness (number of ribotypes), evenness, Shannon diversity and Simpson's diversity indices of bacterial community in rhizosphere soil from different sampling sites based on T-RFLP analysis.

Sampling site	Number of ribotypes			Evenness index			Shannon diversity			Simpson's diversity		
	*Msp*I	*Hha*I	*Rsa*I	*Msp*I	*Hha*I	*Rsa*I	*Msp*I	*Hha*I	*Rsa*I	*Msp*I	*Hha*I	*Rsa*I
300 m above outfall	61	31.5	8.5	0.9	0.92	0.75	3.69	3.17	1.61	0.98	0.96	0.72
Pool above outfall	16	21	10	0.61	0.55	0.62	1.68	1.68	1.44	0.7	0.69	0.68
Pool below outfall	4	10.5	1.5	0.79	0.74	0.25	1.07	1.74	0.18	0.59	0.72	0.1
2000 m below outfall	22.67	40.67	8	0.84	0.86	0.51	2.6	3.16	0.91	0.88	0.94	0.48

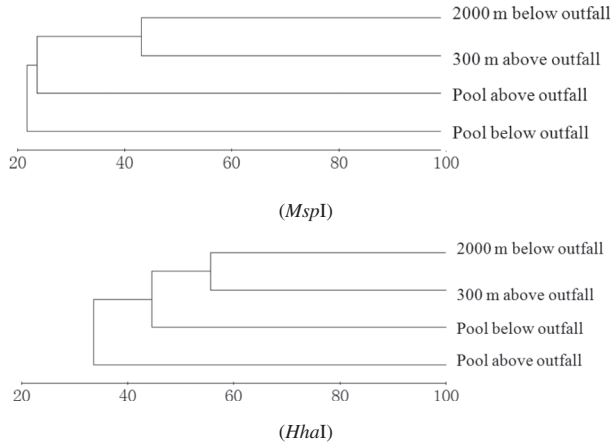

Fig. 2 – Dendrogram of hierarchical cluster analysis based on the T-RFLP profiles of *T. angustifolia* rhizosphere soil samples digested with two restriction (*Msp*I, *Hha*I) enzymes.

remaining 392 were positive clones. The remaining sequences were grouped into 48 different OTUs with >97% sequence identity by Muther soft. These sequence data have been submitted to the GenBank databases under the accession number KR095234–KR095278. Additionally, the calculated coverage of the clone library was 97.95%. For the downstream library, two hundred and seventy-five individual sequences derived from 320 positive clones were verified by colony PCR and submitted to GenBank (accession no.: KR095199–KR095233), and the calculated coverage of the clone library was 97.82%.

Phylogenetic analysis of all the sequences in the upstream sample revealed that the majority of clones were affiliated with Proteobacteria (259 clones, 66.04%) and Bacteroidetes (57 clones, 14.55%). The other clones belonged to Acidobacteria (14 clones, 3.85%), Verrucomicrobia (12 clones, 3.06%), Gemmatimonadetes (11 clones, 2.81%), Firmicutes (4 clones, 1.03%) and uncultured bacterium (35 clones, 8.93%). Analysis based on the class level showed that the most abundant taxa of Proteobacteria were β-Proteobacteria (118 clones, 30.14%) and ε-Proteobacteria (65 clones, 16.58%), followed by δ-Proteobacteria (40 clones, 10.12%), α-Proteobacteria (20 clones, 5.11%) and γ-Proteobacteria (16 clones, 4.09%) in the Proteobacteria phyla. In the downstream sample the most abundant phyla was also Proteobacteria (137 clones, 49.82%), followed by Bacteroidetes (45 clones, 16.36%). The other clones belonged to Firmicutes (24 clones, 8.73%), Chloroflexi (21 clones, 7.64%), Acidobacteria (16 clones, 5.82%), Verrucomicrobia (8 clones, 2.91%) and Gemmatimonadetes (4 clones, 1.45%). In the Proteobacteria phyla, the most abundant taxa of Proteobacteria were β-Proteobacteria (50 clones) and γ-Proteobacteria (45 clones), the proportion of which was 18.18% and 16.36% respectively, followed by δ-Proteobacteria (27 clones, 9.82%), α-Proteobacteria (10 clones, 3.64%) and ε-Proteobacteria (5 clones, 1.82%). Details of all OTUs in the two clone libraries are listed in Tables S1 and S2. Overall, there were some differences among upstream and downstream samples in the distribution of the taxonomic groups. As shown in the Fig. 4, RWTP effluent markedly reduced the relative abundance of Proteobacterial sequences, and the decrease of which was driven mainly by a decrease in the abundance of β-Proteobacteria and ε-Proteobacteria, while an increase in the relative abundance of γ-Proteobacteria at the downstream samples. Additionally, there was a striking increase in the relative abundance of Firmicutes and Chloroflexi sequences.

β-Proteobacteria was the most abundant class in the phylum of Proteobacteria from the upstream and downstream sediments, while the relative abundance of β-Proteobacteria was markedly higher in the upstream without reclaimed water influence. In the upstream, 118 clones in the class β-Proteobacteria comprising 18 OTUs were mainly related to five orders of bacteria, which included Burkholderiales, Rhodocyclales, Hydrogenophilales, Methylophilales and Nitrosomonadales, the remainder belong to unclassified. However, there are only four OTUs, which comprised 50 clones, were found in the downstream sample. In detail, Burkholderiales was the most abundant order in the class of β-Proteobacteria in the upstream and downstream samples, and the clones in the two clone libraries exhibited the higher levels of similarity to Burkholderiales. The members of Rhodocyclales were highly enriched in the upstream sample,

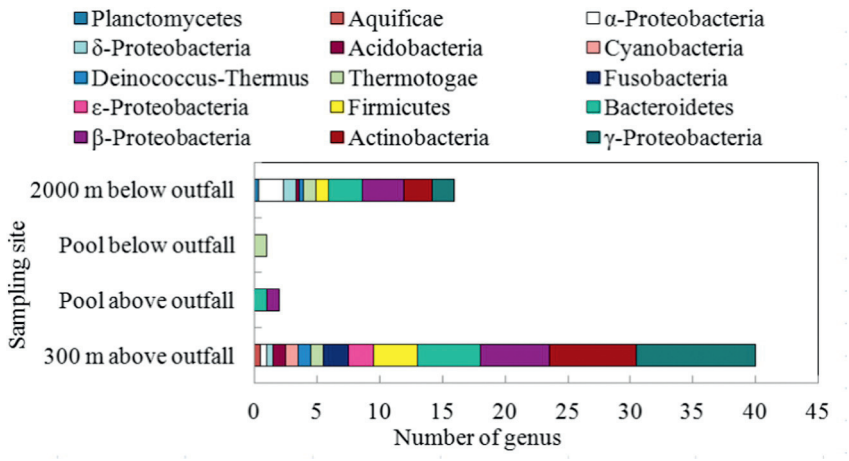

Fig. 3 – Barchart of bacterial community diversity in Typha rhizosphere classification based on phylum level.

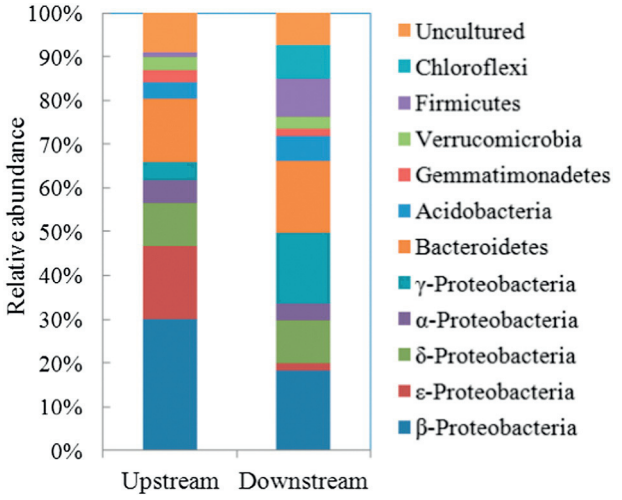

Fig. 4 – Phylogenetic distribution of the OTUs in the two clone libraries. Relative read abundance of different bacterial phyla within the different communities. Sequences that could not be classified into any known group were assigned as 'Uncultured'.

but were not detected in the downstream. Some studies have shown that Rhodocyclales played an important role in organic pollutant and nitrogen compound removal (Xia et al., 2012). The relative abundance of Hydrogenophilales was markedly increased in the downstream sample. In the upstream, 12 clones comprising two OTUs associated with heavy metal resistance from polluted sediment had high similarity with *Thiobacillus* sp., each OTU was respectively isolated from Hg polluted sediment and leachate sediment (Regier et al., 2012; Liu et al., 2011). In the downstream, one OTU of a total of 18 clones were highly similar to Hydrogenophilaceae from the anaerobic environment in tar oil contaminant plume (Winderl et al., 2008).

ε-Proteobacteria was the secondary abundant class in the phylum of Proteobacteria in the upstream, and the obvious decrease of ε-Proteobacteria abundant happened in the downstream. The most abundant genus was *Sulfuricurvum* sp. RIFRC-1 in the study area. A few studies have been reported, bacteria belonging to the genus of *Sulfuricurvum* in the family of Helicobacteraceae are usually a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium (Kodama and Watanabe, 2004).

The relative abundance of γ-Proteobacteria was markedly increased in the downstream, especially Sinobacteraceae and Xanthomonadaceae. In the downstream, forty-two clones in the class γ-Proteobacteria comprising three OTUs were related to two families (Sinobacteraceae and Xanthomonadaceae) and these included three genera (*Steroidobacter*, *Xanthomonas* and *Lysobacter*). A majority of the sequences (26 clones) exhibited high similarity to bacteria of genus *Steroidobacter* sp., which was the most abundant genus in the class of γ-Proteobacteria in the downstream sample and originally isolated from Hg polluted sediment (Regier et al., 2012). Sixteen clones were closely related to Xanthomonadaceae and belonged to genera *Xanthomonas* and *Lysobacter*. It has been reported that *Xanthomonas* sp. is an active participant in the degradation of contaminants, such as petrochemical compounds (Hernandez-Raquet et al., 2006; Lafortune et al., 2009). Additionally, a few studies have been reported that the microorganisms belonging to the order Xanthomonadales are linked normally to plants diseases (Bashan, 1986; Alfano and Collmer, 1996).

Among non-Proteobacteria, a higher relative abundance of Firmicutes and Chloroflexi sequences was detected at the downstream site. In Firmicutes, 24 clones grouped into *Clostridium* sp. in the downstream, including three OTUs, were closely related to three different species of *Clostridium*. Each OTU was respectively originally isolated from leachate sediment, a contaminated aquifer and hexavalent chromium contaminated soil. In Chloroflexi, a total of 15 clones comprise three OTUs in the family of Anaerolineaceae. Among them, the most dominant OTU, which comprised 11 clones, was very similar to Anaerolineaceae, which is originally isolated from Ajka red mud contaminated soils with arsenic, chromium, molybdenum and Vanadium during progressive anoxia. The other 10 clones were related to the genera *Longilinea* and *Anaerolineas*.

3. Discussion

Concerning the bacterial community in the tissue of *T. angustifolia* in the wetland with RWTP effluent supplement, so far, a few studies have been performed. Li et al. (2011) and Guo et al. (2015) examined the endophytic bacterial diversity in the root of *T. angustifolia* by using 16S rRNA clone library analysis. Both their results showed that Proteobacteria were found to be the predominant phylum and β-Protebacteria were the most abundant class in the phylum of Proteobacteria, followed by γ-Proteobacteria, δ-Proteobacteria, α-Proteobacteria and ε-Proteobacteria, which were in agreement with the study in the downstream sample. Although those studies were performed in the wetland with RWTP effluent supplement, they didn't compare with other sample for this kind of special water quality. This paper takes the upstream sample as a comparison, trying to evaluate the impact of RWTP effluent on bacterial function and community structure in the *T. angustifolia* rhizosphere sample.

Using a combination of terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of the 16S rRNA gene sequences, we evaluated the impact of RWTP effluent on the ecology of bacterial communities in the *T. angustifolia* rhizosphere sample. The result of community diversity analysis showed that bacterial richness, evenness and diversity decreased dramatically with the increase of the reclaimed water interference intensity. Differences in bacterial community characteristics were strongly linked with the water quality characteristics and physicochemical properties of the sediment. However, the detected eutrophication indicators (TN, TP, ORP, TOC, NH_4^+-N, pH, Sal, TDS, DO and Chl-*a*) did not show the corresponding variation. For example, when comparing the above indicators in the pool below outfall with that in the downstream, we would find that they were either nearly the same values or decrease. However, the bacterial community diversity was markedly increased in the downstream. Additionally, in contrast to the upstream, the richness, evenness and diversity of bacterial community in

the pool below was extremely low, indicating that there may have been a secondary effect from the RWTP effluent affecting the bacterial community characteristic near the outfall. It is well known that RWTPs are a significant source of disinfection byproducts, antibiotics, heavy metals, pharmaceuticals and pesticides, which may produce detrimental effects on microbial communities (Drury et al., 2013; Proia et al., 2013). Based on these observations, we expected that microbial diversity may be affected by some toxic compounds containing RWTP effluent, which might have contributed to a decrease in bacterial diversity from near the outfall sites, while the recovery of wetland ecosystem by the wetland plant rhizosphere microorganism associated with plants contributed to the rise in bacterial diversity and species richness at the downstream location (Stottmeister et al., 2003). This result was accordant with published study on the shifts in sediment bacterial communities caused by RWTP effluent (Ma et al., 2014).

Rhizophere bacterial community is the main force involved in the degradation of pollutants and playing an important role in maintaining the balance of wetland ecosystem and achieving ecological purification (Feng et al., 2012), while the bacterial biomass, bacterial diversity, bacterial community structure and composition may also be affected by pollutants containing RWTP effluent. A higher relative abundance of γ-Proteobacteria, Firmicutes and Chloroflexi and a lower proportion of β-Proteobacteria and ε-Proteobacteria sequences was detected at the downstream site compared to the upstream site. The higher proportion of Firmicutes and Chloroflexi sequences reflects the impact of RWTP effluent, because some members of these phyla have been proposed as indicators of fecal pollution, and they were commonly abundant in anaerobic wastewater treatment systems (Riviere et al., 2009). Previous studies have also demonstrated that the class Clostridia, which was dominant among the Firmicutes at the downstream point, has frequently been detected within urban wastewater systems (Vierheilig et al., 2013).

In this study, a total of 21.7% of the clones in the upstream clone library may be closely related to the biological cycle of nitrogen (nitrogen fixation, ammonification, nitrification and denitrification) in wetland systems. The majority of the sequences belonged to *Sulfuricurvum* sp. RIFRC-1, *Burkholderia* sp. S1-40 and *Rhodocyclus* sp. W4S68, with the remainder spread among Rhodocyclaceae, Comamonadaceae, and other minor groups. Among them, *Sulfuricurvum* sp. RIFRC-1 could translate nitrate into nitrite (Kodama and Watanabe, 2004), *Burkholderia* sp. S1-40 had a strong denitrogenation ability (Yoshida et al., 2012) and *Rhodocyclus* sp. W4S68 was capable of reducing nitrate (Hougardy and Klemme, 1995). What's more, a total of 19.27% of the clones in the downstream clone library may be closely related to the biological cycle of nitrogen, such as *Burkholderia* sp. S2-86, *Steroidobacter* sp. (Fahrbach et al., 2008), *Geobacter* sp. KB-11 (Lovley et al., 1993), *Phenylobacterium* sp. b2-194 (Lingens et al., 1985) and *Sulfuricurvum* sp. RIFRC-1. For the biological cycle of phosphorus, a total of 6.13% clones in the upstream clone library may be closely related to the biological cycle of phosphorus in wetland systems, mainly including *Rhodocyclus* sp. W4S68 (Ahn et al., 2001), *Dechloromonas* sp. 4y-107 (Zhang et al., 2011) and *Gemmatimonas* sp. Wbfc97 (Yang et al., 2012). In contrast to the upstream, about 6.9% clones were related to the biological cycle of phosphorus in the downstream, belonging to Bacteroidetes and Gemmatimonadetes. In the upstream clone library, about 25.04% of the bacterial community groups showed a close relationship with carbon recycling, such as *Ramlibacter* sp. W1.09-2 (Heulin et al., 2003), *Rhodocyclus* sp. W4S68 (Pfennig, 1978), *Methylobacillus* sp. R15-12 (Akinori et al., 2002), *Tolumonas* sp. R40-25 (Fischer-Romero et al., 1996), *Prolixibacter* sp. (Holmes et al., 2007) and *Geothrix* sp. MOBA74_25m (Coates et al., 1999). Approximately 26.55% of the bacterial community groups were closely related to carbon recycling in the downstream clone library. The most dominant genera were *Clostridium* spp. (Zhao et al., 2008), *Desulfobacula* sp. LU2-210 and *Phenylobacterium* sp. b2-194, followed by *Lysobacter* sp. T313C8, *Geobacter* sp. KB-11 and *Alkaliflexus* sp. Pad-81. A total of 19.65% clones in the upstream clone library may be closely related to the biological cycling of sulfur in wetland systems. Some principle members of the genera *Sulfuricurvum* sp., *Desulfomicrobium* sp. (Azabou et al., 2007), *Desulfuromonas* sp. (Pfennig, 1978) and *Thiobacillus* sp. (Visser et al., 1997) all played an important role in the sulfur cycle in wetland. A total of 20.37% of the clones in the downstream clone library may be closely related to the biological cycling of sulfur in wetland systems, main including *Clostridium* spp. (Zhao et al., 2008), *Desulfomicrobium* sp., *Desulfobacula* sp. LU2-210 and *Geobacter* sp. KB-11 in δ-Proteobacteria and *Sulfuricurvum* sp. RIFRC-1 in ε-Proteobacteria.

Based on the above analysis, we could conclude that the bacterial community function structure characteristics didn't strikingly change for nitrogen cycle, phosphorus cycle and carbon cycle between the upstream and downstream. This is the reason that the nutrient indicators TN, TP and TOC did not change significantly between the upstream and downstream. Based on the above conclusion, it is further confirmed that there may have been a secondary effect from the RWTP effluent affecting the bacterial community characteristic, such as heavy metals.

A large number of studies have shown that reclaimed water contains residue of halogenated hydrocarbons, heavy metal, antibiotics and other drugs contaminate groundwater when it replenishes river channels, which may produce detrimental effects on microbial communities (Drury et al., 2013). In the case of those pollutants, substantial changes happened in bacterial structure and composition. This study showed that about 9.45% clones were closely associated with the resistance of antibiotics containing environments in the upstream sediment, with dominant groups of *Comamonas* sp. R15-4, *Rhodocyclus* sp. W4S68 and *Methylobacillus* sp. R15-12 in β-Proteobacteria and *Tolumonas* sp. R40-25 in γ-Proteobacteria. Approximately 7.64% clones in the downstream clone library may be closely related to the resistance of antibiotics in wetland systems, which belong to Burkholderiales bacteria. For the bacterial community related to the resistance of halogenated hydrocarbons in wetland systems, a total of 3.33% of the clones in the downstream clone library may be closely associated with the resistance of halogenated hydrocarbons in wetland systems and 3.6% clones in the downstream clone library. It is noteworthy that a total of 6.13% of the clones in the upstream clone library might be specifically associated with heavy metal containing environments. While 14.9% clones in the downstream clone library may be closely related to the heavy metal in wetland systems, some

heavy-metal resistant bacteria *Steroidobacer* sp., *Geobacter* sp. KB-11 and *Clostridium* sp. XT40, which were found at the downstream site, were not detected or only rarely detected at the upstream site. Obviously, the relative abundance of heavy-metal resistant bacteria striking changed between the upstream and the downstream, and the downstream sample corresponded to higher abundance of these bacteria. Combined with the analysis results of physicochemical properties of *T. angustifolia* rhizosphere soil, we would expect that bacterial diversity may be affected by the heavy metal.

It is well known that RWTP effluent is rich in pathogenic bacteria, which may produce detrimental effects on humans (Toze, 2006). Compared to the upstream, the pathogenic bacteria were markedly increased in the downstream rhizosphere soil. Approximately 12.37% clones of pathogenic were detected in the downstream clone library, with dominant groups of *Clostridium* sp. (Butel et al., 1998) and *Xanthomonas* sp. burs_22 (Li et al., 2008). However, few potentially pathogenic bacteria were detected in the upstream. On the other hand, Plant growth-promoting rhizobacteria (PGPR) were markedly decreased in the downstream sediment since reclaimed water supplement. About 27 clones of PGPR were obtained from the upstream clone library, including *Burkholderia* sp. (Estrada-De Los Santos et al., 2001) and *Rhizobium* sp. While a total of 7 PGPR clones were detected in the downstream, which belong to *Burkholderia* sp.

4. Conclusions

The combination of T-RFLP and 16S rRNA gene library techniques was a powerful approach for understanding the diversity and community structure of *T. angustifolia* rhizosphere bacteria in the constructed Beijing Mayu Wetland. In our survey, RWTP effluent could markedly change the rhizosphere bacterial community structure characteristics, resulting in decrease in bacterial community richness, evenness and diversity from downstream site. Rhizosphere bacterial community function may be changed between the upstream and downstream locations, simultaneously. These changes may be caused by heavy metals and other drugs contaminate containing RWTP effluent. The relative abundance of bacteria closely related to the resistant of heavy-metal was markedly changed, while the bacterial community function structure characteristics didn't change significantly for nitrogen cycle, phosphorus cycle, carbon cycle and sulfur cycle between the upstream and downstream sites. Additionally, the pathogenic bacteria were markedly increased in the downstream rhizosphere sample because of RWTP effluent supplement, while PGPRs were markedly decreased in the downstream sample. Though, of course, all these conclusions will be further confirmed with corresponding data based on analyses for bacterial resistance to heavy-metals, the presence of PGPR features, detection of respective functional genes in the metagenomic DNA, water quality, soil physicochemical properties, *etc.*

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.06.022.

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