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Seasonal and spatial dynamics of denitrification rate and denitrifier community in constructed wetland treating polluted river water

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ABSTRACT

Denitrification community in wetland plays an important role in nitrogen removal. The present study investigated the seasonal and spatial dynamics of denitrification rate and *nirS*-denitrifier communities and the potential influential factors in a large wetland system treating polluted river water. Wetland denitrification rate and the abundance, richness, diversity and composition of *nirS*-denitrifier community were found to vary with season and sampling site. Both wetland denitrification rate and denitrifier community were related to plant type. Wetland soils and sediments differed greatly in either denitrification rate or denitrifier community structure. Wetland generally had lower denitrification rate and denitrifier abundance in summer than in spring and winter. Denitrification rate showed no direct correlation to denitrifier abundance but was positively correlated to denitrifier diversity. Denitrification rate could be mediated by denitrifier community structure. Moreover, Spearman rank correlation analysis suggested that denitrification rate was significantly correlated to sediment/soil ammonia, nitrate, nitrite, total phosphorus and pH, while denitrifier abundance was significantly correlated to total phosphorus and temperature. Nitrite, total nitrogen, total organic carbon, and the ratio of total organic carbon to total nitrogen showed significant correlations with wetland denitrifier diversity, while ammonia, nitrate, total nitrogen and total phosphorus might have important roles in shaping wetland denitrifier community structure. In addition, for each wetland sediment or soil, 0.8–46.2% of the retrieved *nirS* sequences could be related to the sequences from cultivated denitrifiers. *Dechloromonas*-like denitrifiers were more abundant in wetland sediments than in wetland soils.

1. Introduction

Constructed wetlands (CWs) have found increasing applications for the treatment of polluted surface water (Martin et al., 2013; Tanaka et al., 2015; Zhi et al., 2015; Ge et al., 2016; Long et al., 2016, 2017). In CWs, microorganisms are believed to play crucial roles in removing or transforming organic and inorganic pollutants (Martin et al., 2013; He et al., 2016; Ibekwe et al., 2016; Wu et al., 2016a, 2016b, 2016c; Sanchez, 2017). Denitrification is a facultative anoxic respiration process through which nitrate and nitrite are reduced to nitric and nitrous oxides, and finally to dinitrogen gas (N_2) in consecutive reaction steps catalyzed by different enzymes (Zumft and Körner, 1997). Denitrification in CWs is a key process of removing nitrogen and controlling eutrophication in downstream aquatic ecosystems (Chyan et al., 2016). Nitrogen compounds can be effectively removed in CWs that are used to treat polluted surface water (Li et al., 2008; Martin et al., 2013). Denitrification can be achieved by phylogenetically unrelated bacterial groups (Zhou et al., 2016), and denitrifier assemblages can show considerable variability in their physiology (Peralta et al., 2010). Hence, the shift in environmental variables can influence denitrifying microbial physiology, denitrification rate and denitrifier community composition (Peralta et al., 2010). However, limited information exists on temporal and spatial dynamics of denitrifier communities in CWs treating surface water and the associated environmental factors. Several previous studies suggested that denitrifier communities in wetland sediments or soils might be mediated by a variety of factors, such as plant harvest management (Tanaka et al., 2015), nitrification rate (Tanaka et al., 2015), dissolved organic carbon (DOC) level in water (Zhi et al., 2015), water regime (Ligi et al., 2014), soil pH (Ligi et al., 2014), wetland type (Ligi et al., 2014), soil nitrate nitrogen content (Ligi et al.,

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2014), and vegetation presence (Song et al., 2012). However, how multiple factors collectively shape the distribution of denitrifier community in CWs remains unclear. Moreover, the interplay between denitrification rate and denitrifier community in wetland ecosystem remains in debate. A few previous studies indicated a close link of denitrification rate with denitrifier community abundance or/and composition in natural wetlands (Ma et al., 2011; Hu et al., 2014; Yang et al., 2015) and even in a free water surface flow CW (FWSF-CW) (Kjellin et al., 2007), while other studies suggested no or poor links between denitrification rate and denitrifier community in FWSF-CWs (Song et al., 2012; Tanaka et al., 2015).

Previous investigations on wetland denitrifier diversity and composition were based on low-profiling molecular biology approaches, such as clone library analysis (Gao et al., 2016), terminal-restriction fragment length polymorphism (TRFLP) (Baneras et al., 2012; Hu et al., 2014; Li et al., 2015), and denaturing gradient gel electrophoresis (DGGE) (Kjellin et al., 2007; Tanaka et al., 2015). The investigations using low-profiling molecular biology approaches could underestimate microbial diversity in complicated ecosystems, which can narrow the understanding of wetland denitrifier community. In contrast, high-throughput sequencing can contribute to more extensive and systematical investigations on denitrifier populations in complicated ecosystems. High-throughput sequencing analysis has found applications in characterizing denitrifier community diversity and composition in a variety of ecosystems, such as river periphytic biofilms (Kalscheur et al., 2012), soil (Palmer et al., 2012), lake sediment (Saarenheimo et al., 2015; Mao et al., 2017), reservoir sediment (Zhou et al., 2016), and lake water (Mao et al., 2017). However, information about the application of high-throughput sequencing to characterize wetland denitrifier community is still lacking. In addition, *nirS* gene, encoding nitrite reductases (Nir), has been one of the commonly used biomarkers to detect wetland denitrifier communities (Song et al., 2012; Li et al., 2015; Tanaka et al., 2015; Gao et al., 2016). Therefore, the overall objective of the present study was to investigate temporal and spatial dynamics of denitrification rate and denitrifier community in a FWSF-CW treating polluted river water and the associated environmental factors. The diversity and composition of wetland denitrifier communities were characterized using Illumina MiSeq high-throughput sequencing, while denitrifier rate and abundance were estimated by acetylene inhibition technique and quantitative PCR (q-PCR) assay of *nirS* gene, respectively.

2. Materials and methods

2.1. Study sites and sampling

During spring (March), summer (August) and winter (December) in 2016, wetland sediments (at sites A–F) and soils (at sites G and H) (0–10 cm depth) in triplicate were obtained from eight different locations (25°56′42″–25°57′11″ N, 100°6′0″–100°6′9″ E) in a large FWSF-CW. Sediments were collected using Kajak tube core samplers (KC Denmark A/S, Holmbladsvej, Silkeborg, Denmark). Sediments and soils were placed into sterile glass containers, sealed, and transported back to the laboratory on ice. The FWSF-CW, established about eight years ago, had a surface area of about 0.47 km^2 and an average water depth of 1.5 m, and it was mainly designed to treat the water of Luoshi River entering Lake Erhai in southwest China at an average flow of 3.5 m^3/s. Luoshi River was mainly polluted by agricultural runoff and rural domestic wastewater. The FWSF-CW could effectively abate total nitrogen (TN), with an average TN removal rate of 43.2% (Li et al., 2017). The annual average air temperature and precipitation in local region (having a subtropical monsoon climate) were about 15.7 °C and 1000 mm, respectively. Sites A–F were permanently inundated, and the dominant wetland plant species in these sites were water hyacinth (25°57′5.8″ N, 100°6′7.7″ E), reed (25°57′5.3″ N, 100°6′6.7″ E), watermilfoil (25°54′34.3″ N, 100°6′0.2″ E), penny grass (25°56′52.9″ N, 100°6′1.3″ E), duckweed (25°56′49.4″N, 100°6′3.3″ E), and water-lily (25°56′41.8″ N, 100°6′2.5″E), respectively. Site G (located at the wetland center highland) and site H (located at the wetland edge) were only inundated during summer and autumn, and these sites were planted with alfalfa (25°57′10.9″N, 100°6′9.3″ E) and cattail (25°57′2″ N, 100°6′1.7″ E), respectively. All sediment and soils samples were collected from rhizosphere zones. In accordance with the previous study (Li et al., 2017), the collected wetland sediment/soil samples were coded as A–H, corresponding to the sampling site, and SP, SU, or WI, corresponding to the sampling period (spring, summer, or winter, respectively). The sediment sample at site B during winter was not collected because of inaccessibility; thus, no sample was coded "BWI." The physicochemical properties of wetland soils and sediments were described in detail in the previous study (Li et al., 2017). pH, temperature, oxidation and reduction potential (ORP), total phosphorus (TP), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), ammonia nitrogen (NH_4^+-N), total nitrogen (TN), total organic carbon (TOC), and the ratio of TOC to TN (C/N) were 7.06–7.99, 10.6–27.8 °C, −59.9–5.6 mv, 46.69–1411.36 mg/kg, 0.01–1.26 mg/kg, 0.42–187.61 mg/kg, 1.29–34.28 mg/kg, 647.36–2564.06 mg/kg, 5.10–44.85 g/kg, and 3.9–21.5, respectively (Li et al., 2017).

2.2. Denitrification rate

Each replicate sediment/soil sample was individually subjected to the determination of denitrification rate. Denitrification rates of wetland sediments or soils were determined using the acetylene inhibition procedure (Yang et al., 2015). Approximately 150 mL sediment (or soil) sample was placed in anoxic bottle (with an inner diameter of 57 mm and a total volume of 335 mL). Anoxic condition in each bottle was achieved by purging N_2 gas for 30 min. Before the measurement, wetland sediment was filled with overlying water and was incubated for 12 h at ambient temperatures before the appearance of brown lamina. Overlying water was then carefully discarded, and helium gas was injected into the bottle in order to maintain anoxic condition. Pure C_2H_2 gas was injected to the headspace of each bottle (10% v/v). In this study, the experiments were performed in triplicate. Gas samples (185 mL) were analyzed using an Agilent 7890A gas chromatograph equipped with a 5 Å molecular sieve column and a Ni^{63} electron capture detector (Smith et al., 1978) to measure N_2O level in headspace gas after an 8-h-incubation at 120 rpm at ambient temperature (about 15 °C). Highly pure nitrogen was used as carrier gas at a flow rate of 1 mL/min. The split ratio was set at 8:1 and the injection volume was 500 μL.

2.3. Molecular analyses

Sediment/soil DNA was extracted with the PowerSoil™ DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA). The preliminary study indicated that the density of *nirK*-denitrifier in the studied FWSF-CW was below q-PCR detection (data not shown), so the present study only monitored the density of *nirS*-denitrifier.

Each replicate sediment/soil DNA sample was individually subjected to q-PCR assay. The density of *nirS* gene in wetland sediment/soil was estimated by q-PCR assay using primers nirScd3aF (5′-GTSAACGTSAAGGARACSGG-3′)/nirSR3cd (5′-GASTTCGGRTGSGTCTTGA-3′) (Kandeler et al., 2006), following the same amplification conditions reported in the previous study (Mao et al., 2017). A standard curve was generated with 10-fold serial dilutions of standard plasmids harboring the target functional gene. The specificity of the qPCR for each reaction was confirmed by melting curve analysis. The amplification efficiency and the linear regression coefficient were 97% and 0.995, respectively.

For high-throughput sequencing analysis, *nirS* gene was also amplified using primer pair nirScd3aF/nirSR3cd according to the literature (Zhou et al., 2016). The purified PCR products (with QIAquick PCR

purification kit, Qiagen Inc.) from triplicate samples were pooled in equal amounts for Illumina MiSeq sequencing at TinyGene Bio-Tech (Shanghai) Co., Ltd using a HiSeq 2000 system (Illumina Inc., San Diego, CA, USA). Raw reads were deposited in the Sequence Read Archive database (accession number: SRP102967). Paired-end reads were merged with FLASH and low quality sequences were discarded using QIIME (Caporaso et al., 2010). Chimeric composite sequences were detected and deleted using UCHIME (Edgar et al., 2011). Chimeric-free *nirS* gene sequences were grouped into operational taxonomic units (OTUs) based on a 3% dissimilarity cutoff and α-diversity indices (Chao1 richness and Shannon diversity) were then obtained using UPARSE (Edgar, 2013). Moreover, phylogenetic analysis of *nirS* OTUs and their reference sequences reported in the NCBI GenBank database was performed with the MEGA software 6.0 (Tamura et al., 2013) based on neighbor-joining analysis of 1000 resampled datasets was further visualized using the online tool Interactive Tree Of Life (iTOL) v3 (http://itol.embl.de) (Letunic and Bork, 2016). To compare the difference of *nirS*-denitrifier community compositions among wetland samples, weighted UniFrac distance was generated and then hierarchical clustering based on weighted pair group method with arithmetic mean (WPGMA) was carried out using *R* (version i386, 3.3.0).

The differences in denitrification rate or *nirS* gene number among sampling sites and seasons were compared at a significance level of 0.05 by one-way analysis of variance. The links of wetland sediment/soil physicochemical parameters and denitrification rate with *nirS*-denitrifier abundance, richness and diversity were determined based on Spearman's rank correlation analysis using the software SPSS 20.0 (IBM, Armonk, NY, USA). Redundancy analysis (RDA) using Monte Carlo tests was applied to find the correlations between *nirS*-denitrifier community composition and environmental parameters using the software CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA). The relative abundance of *nirS* sequences in each OTU was assigned as species input, whereas sediment/soil physicochemical parameters was used as environmental input.

3. Results

3.1. Denitrification rate

Denitrification rates in wetland soils varied from 4.66 to 53.84 μmol N_2O/m^2. h, while wetland sediments had denitrification rates of 0.12–13.88 μmol N_2O/m^2. h (Fig. 1). At sites G and H, wetland soils showed higher denitrification rate during spring than during summer and winter ($P < 0.05$). At site G, the soil collected during winter also showed significantly higher denitrification rate than the one collected during summer ($P < 0.05$). At sampling sites A, C, and D, significant differences in sediment denitrification rate were observed among three seasons ($P < 0.05$). At sites B, E, and F, the sediment collected during summer illustrated much lower denitrification rate than the corresponding one collected during spring or winter, although no statistical significance was found. Wetland sediments and soils tended to have lower denitrification rate during summer than during other seasons. However, at sites A, F, G, and H, the sediment (or soil) collected during spring had higher denitrification rate than the corresponding one collected during winter, while an opposite trend was found at sampling sites C, D, and E. This suggested that the temporal change pattern of wetland denitrification rate was site-specific.

A noticeable spatial variation of denitrification rate in the studied CW occurred during three seasons. During spring, wetland soils displayed much higher denitrification rate than wetland sediments ($P < 0.05$), and the sediment at site A also showed significantly higher denitrification rate than the ones at sampling sites C, D, E, and F ($P < 0.05$). During summer, the wetland soil at site H had significantly higher denitrification rate than the one at site G and all wetland sediments (at sites A–F) ($P < 0.05$). Denitrification rate in the wetland soil at site G was also much higher than those in wetland sediments, although no statistical significance was observed. In addition, during winter, wetland soils showed much higher denitrification rate than wetland sediments ($P < 0.05$), and significant differences in denitrification rate also existed among sediment samples ($P < 0.05$).

3.2. Denitrifier abundance

The density of *nirS* gene in wetland soils varied from 1.52×10^9 to 1.15×10^{10} copies per gram dry soil, while wetland sediments contained 7.39×10^9–1.91×10^{10} *nirS* gene copies per gram dry sediment (Fig. 2). At each site (except site B), significant differences of *nirS* gene density existed among the samples collected during spring, summer and winter ($P < 0.05$), indicating a seasonal variability of denitrifier community size in the studied CW. At most of sampling sites, the sediment/soil sample collected during summer had lower *nirS* gene abundance than the corresponding ones collected during spring and winter. However, at site H, the highest soil *nirS* gene density occurred in summer. Moreover, at site A, *nirS* gene density in the sediment collected during summer was higher than that in the sediment collected during spring but lower than that in the sediment collected during winter. These results also indicated that the seasonal change pattern of wetland denitrifier abundance was site-specific. In addition, during each season, a spatial variation of *nirS* gene density was found in the studied CW.

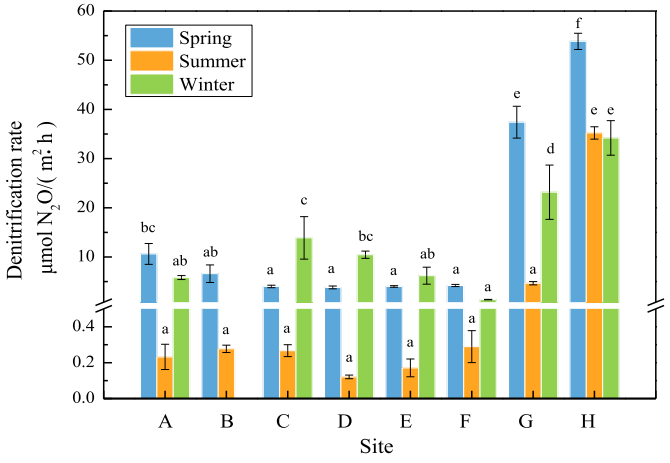

Fig. 1. Denitrification rate of wetland sediments and soils. Values are the average of triplicate samples. Vertical bars indicate standard deviations. Different letters above the columns indicate significant differences in gene abundance ($P < 0.05$).

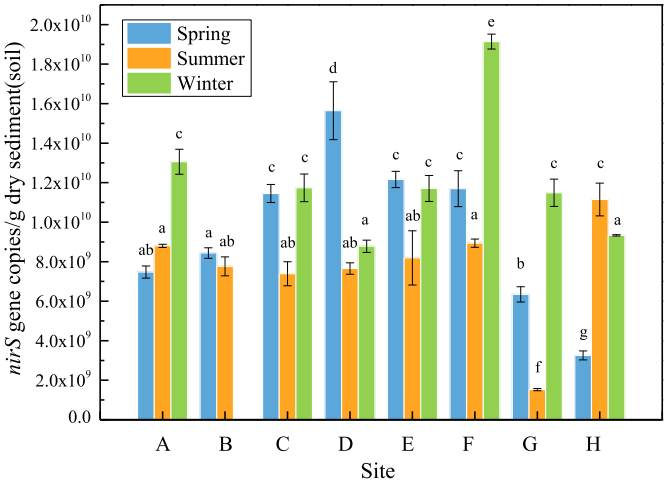

Fig. 2. *nirS* gene abundance of wetland sediments and soils. Values are the average of triplicate samples. Vertical bars indicate standard deviations. Different letters above the columns indicate significant differences in gene abundance ($P < 0.05$).

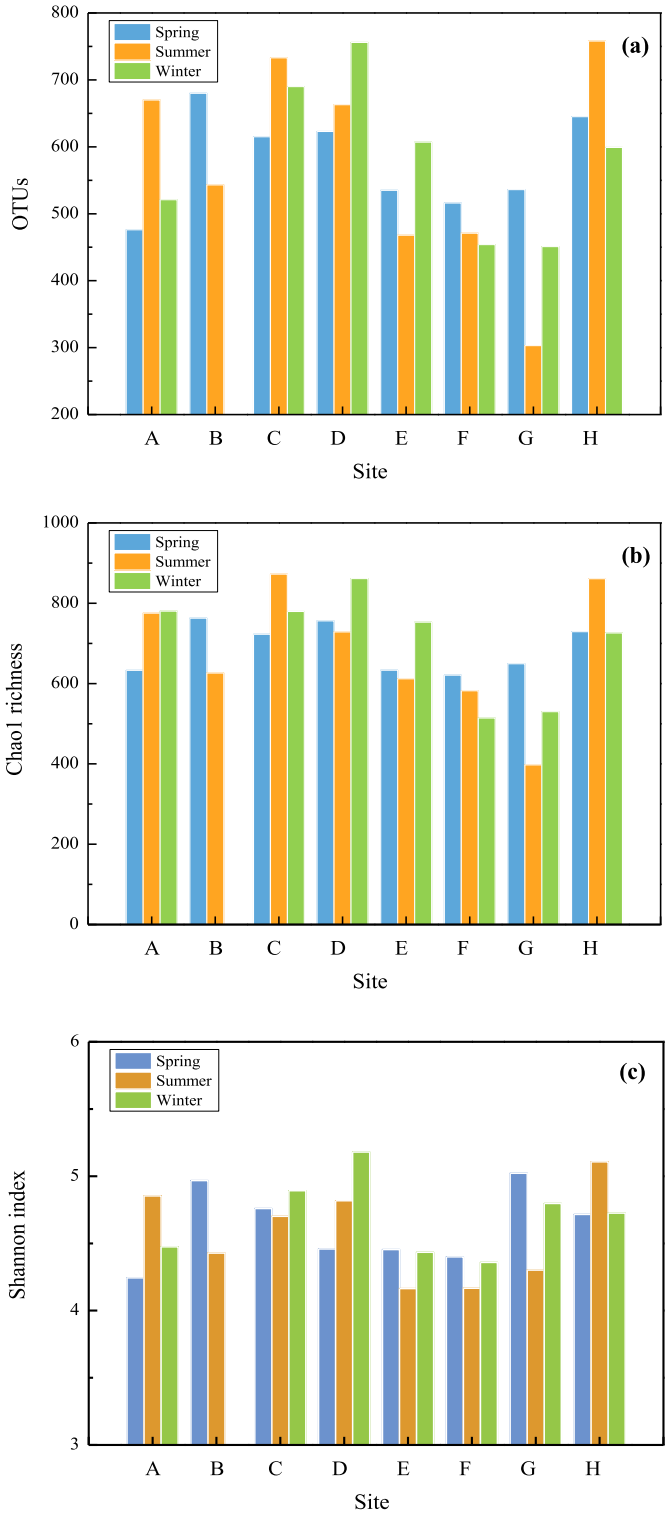

Fig. 3. Wetland sediment or soil *nirS* OTUs (a), Chao1 estimator (b), and Shannon index (c).

3.3. Denitrifier richness and diversity

In this study, to compare *nirS*-denitrifier richness and diversity, the number of valid reads from each wetland sediment/soil sample was normalized to the same sequencing depth (with 24,600 *nirS* sequences). Good's coverage estimator ≥99.5% suggested that OTUs in each *nirS* library had been well captured. Each wetland sediment/soil *nirS* library was comprised of 303–758 OTUs (Fig. 3a). At each sampling site, the number of sediment/soil *nirS* OTUs showed a considerable seasonal change. However, the seasonal change pattern of *nirS* OTU number was also site-specific. At sites A, C, and H, the number of *nirS* OTUs increased from spring to summer but then decreased to winter. In contrast, at sites E and G, the *nirS* OTU number decreased from spring to summer but then increased to winter. At site D, the number of *nirS* OTUs continuously increased from spring to summer and then to winter, whereas an opposite trend was observed at site F. Moreover, during each season, an evident variation of *nirS* OTU number among sediment sampling sites was also observed, and cattail soil had more *nirS* OTUs than alfalfa soil.

Wetland *nirS* Chao1 richness estimator ranged between 398 and 872 (Fig. 3b). Sediment/soil *nirS* Chao1 richness showd an evident seasonal change, depending on sampling site. At site A, *nirS* Chao1 richness continuously increased from spring to summer and then to winter, but an opposite trend occurred at site F. From spring to summer and then to winter, *nirS* Chao1 richness illustrated a rise followed by a decline at sites C and H, but a decrease followed by an increase at sites D, E, and G. Moreover, during each season, a remarkable change of *nirS* Chao1 richness among sampling sites was observed in the studied CW.

The values of wetland *nirS* Shannon diversity index varied from 4.16 to 5.18 (Fig. 3c). Seasonal and spatial changes of *nirS* Shannon diversity also occurred in the CW treating river water. The seasonal change pattern of *nirS* Shannon diversity was site-related. From spring to summer and then to winter, *nirS* Shannon diversity displayed an increase followed by a decline at sites A and H, but a decline followed by a rise at sites C, E, F, and G. In addition, *nirS* Shannon diversity showed a continuous increase with sampling time.

3.4. Clustering analysis of denitrifier communities

The result of WPGMA clustering based on weighted UniFrac illustrated a remarkable spatial change of *nirS*-denitrifier community structure in the studied CW (Fig. 4). Soil samples were distantly separated from sediment samples, whereas alfalfa and cattail soils were also clearly separated. During each season, wetland sediments at all sampling sites were not closely clustered. Moreover, the seasonal variability of *nirS*-denitrifier community structure could also be detected in the CW, depending on sampling site. At sites E and F, the sample collected during summer was distantly separated from those collected during spring and winter. At site C, the sediment sample collected during winter was also distantly separated from those collected during spring and summer. However, at site D, the sediments collected during three seasons were more closely clustered. In addition, the seasonality effect on soil *nirS*-denitrifier community structure was found to be more evident at site G than at site H.

3.5. Phylogenetic analysis of denitrifier communities

In the present study, the major *nirS* OTUs (with the relative abundance of ≥2% in at least one wetland sediment/soil sample) were selected for further phylogenetic analysis. Wetland *nirS*-denitrifier communities could be grouped into a total of 7 distinct clusters (Fig. 5). Cluster 1 included 15 *nirS* OTUs that could be affiliated with the uncultured sequences retrieved from paddy soil and river estuary, lake and wetland sediments. Cluster 1-like *nirS* sequences accounted for a considerable proportion in wetland sediment samples (9.2–30.7%) but became much less abundant in wetland soil samples (0–7.9%) (Table S1). Cluster 2 was composed of 11 *nirS* OTUs that could be grouped with the sequence from a cultivated soil *Ideonella* strain (Wei et al., 2015) and several uncultured sequences from soil and sediment ecosystems. Cluster 2-like *nirS* sequences had a much higher proportion in sample GSU (23.3%) than in other samples (0.2–8.3%). Cluster 3 was a 5-OTU group and its members could be related to the *nirS* sequences from cultivated freshwater *Sulfuritalea* strain (Watanabe et al., 2014) and soil *Cupriavidus* strain (Wei et al., 2015). Cluster 3-like *nirS*

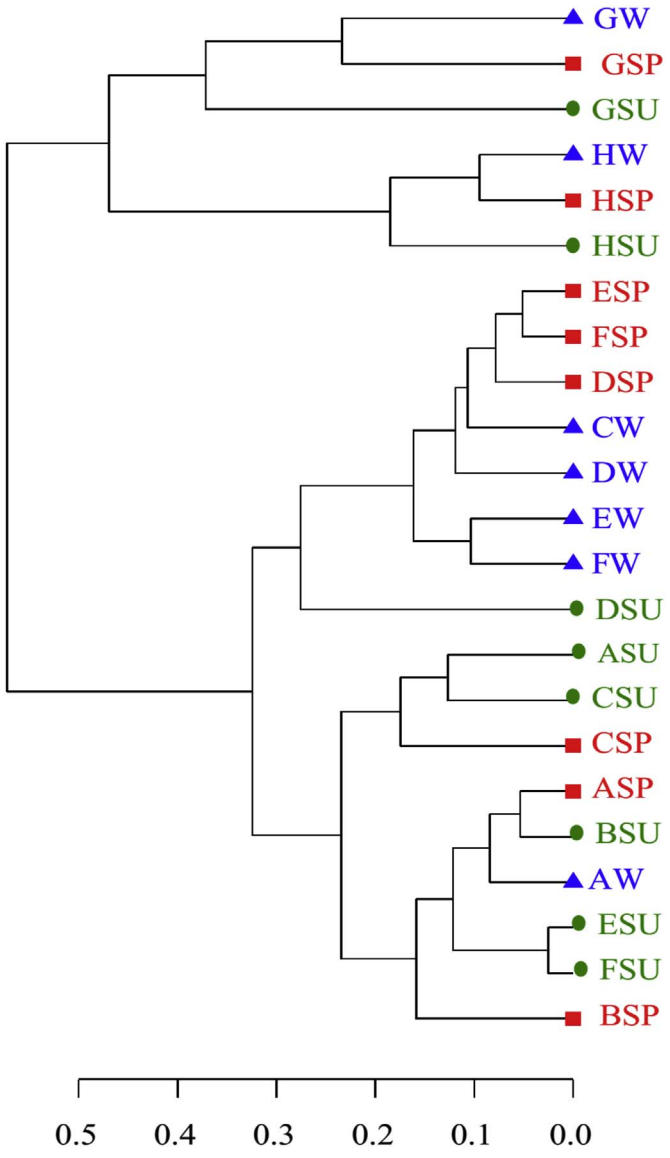

Fig. 4. WPGMA clustering of wetland *nirS*-denitrifier communities based on weighted UniFrac.

sequences generally showed relatively lower proportion in wetland sediments and soils (0–10.9%). Moreover, there were a total of 10 *nirS* OTUs in cluster 4. These OTUs could be related to an uncultured soil *nirS* gene sequence. Cluster 4-like *nirS* sequences displayed much higher proportion in soils (10.5–34.9%) than in sediments (0–7%). Cluster 5 contained 15 *nirS* OTUs that could be affiliated with the sequence from a cultivated *Dechloromonas* strain (Heylen et al., 2006). Wetland sediments (13–39.9%) had much higher proportion of cluster 5-like *nirS* sequences than wetland soils (0.5–4.2%). Cluster 6 and cluster 7 were comprised of 2 and 4 *nirS* OTUs, respectively. The *nirS* OTUs in cluster 6 could be grouped with an uncultured *nirS* gene sequence from activated sludge, while the *nirS* OTUs in cluster 7 were related to an uncultured paddy soil *nirS* gene sequence. Cluster 6-like *nirS* sequences accounted for a low proportion in either sediments (0–6.3%) or soils (0–0.2%). Cluster 7-like *nirS* sequences were also a minor group in both sediments (0–1%) and soils (0–9.2%). In addition, the proportion of *nirS* sequences affiliated with each cluster varied among seasons and sampling sites.

3.6. Influential factors of denitrifier community

Spearman's rank correlation analysis showed that denitrification rate in wetland sediment/soil was positively correlated with the concentrations of NO_3^--N, NO_2^--N, TP and pH ($P < 0.05$ or $P < 0.01$) but negatively with the concentration of NH_4^+-N ($P < 0.01$) (Table 1). Denitrification rate also displayed a positive correlation with denitrifier community diversity ($P < 0.05$), whereas no significant correlation was found between denitrification rate and denitrifier abundance or richness ($P > 0.05$). Wetland denitrifier abundance illustrated negative correlations with the concentration of sediment/soil TP ($P < 0.05$) and temperature ($P < 0.01$). Moreover, wetland denitrifier community richness showed no significant correlation with the determined sediment/soil physicochemical parameters ($P > 0.05$) and but showed a positive correlation with denitrifier diversity ($P < 0.05$). In addition, wetland denitrifier diversity was positively correlated with the concentrations of NO_2^--N, TN and TOC and C/N ratio ($P < 0.05$ or $P < 0.01$).

Wetland environmental factors in the first two RDA dimensions respectively represented 63.6% and 12.9% of the total variance in *nirS* OTU composition (Fig. 6). Wetland sediment/soil physicochemical parameters including TP ($F = 8.475$, $P = 0.001$, 999 permutations), NH_4^+-N ($F = 6.788$, $P = 0.001$, 999 permutations), TN ($F = 5.619$, $P = 0.001$, 999 permutations) and NO_3^--N ($F = 2.096$, $P = 0.045$, 999 permutations) were found to significantly contribute to the denitrifier community–environment relationship.

4. Discussion

4.1. Temporal and spatial changes of wetland denitrification rate

It has been well-documented that denitrification rate can considerably vary among sampling sites and times in sediments or soils of restored and natural wetlands (Baneras et al., 2012; Wang et al., 2013; Hu et al., 2014; Xiong et al., 2015; Peralta et al., 2016), while information about the change of denitrification rate in FWSF-CWs is still limited. Remarkable variations of denitrification rate among sampling sites have been observed in FWSF-CWs treating river water (Song et al., 2012) and wastewater treatment plant effluent (Kjellin et al., 2007). Moreover, two previous studies have shown the temporal change of denitrification rate in FWSF-CWs treating river water (Song et al., 2012; Tanaka et al., 2015). In the current study, wetland samples were collected from eight sites with different plant species in a large FWSF-CW treating polluted river water. Noticeable seasonal and spatial changes of denitrification rate occurred in the studied FWSF-CW. Moreover, the seasonal change patterns of wetland denitrification rate differed among sites with different plants. Therefore, plant type might influence denitrification rate in the FWSF-CW system treating surface water. The influence of plant type on denitrification rate has previously been reported in a natural wetland (Baneras et al., 2012) and in a microcosm wetland (Wang et al., 2014).

So far, the links of denitrification rate in FWSF-CW with wetland physicochemical variables remain poorly known. Denitrification rate in sediment/soil of FWSF-CW was found to be possibly affected by sediment nitrogen (Kjellin et al., 2007) as well as water temperature and NO_3^--N (Song et al., 2012). In this study, the results of Spearman's rank correlation analysis suggested that denitrification rate in the studied FWSF-CW might be collectively regulated by a number of sediment/soil physicochemical variables including NH_4^+-N, NO_3^--N, NO_2^--N, TP, and pH. The potential influences of pH and nitrogen on denitrification rate were also observed in riparian restored and natural wetlands (Peralta et al., 2010; Xiong et al., 2015). Another previous study further

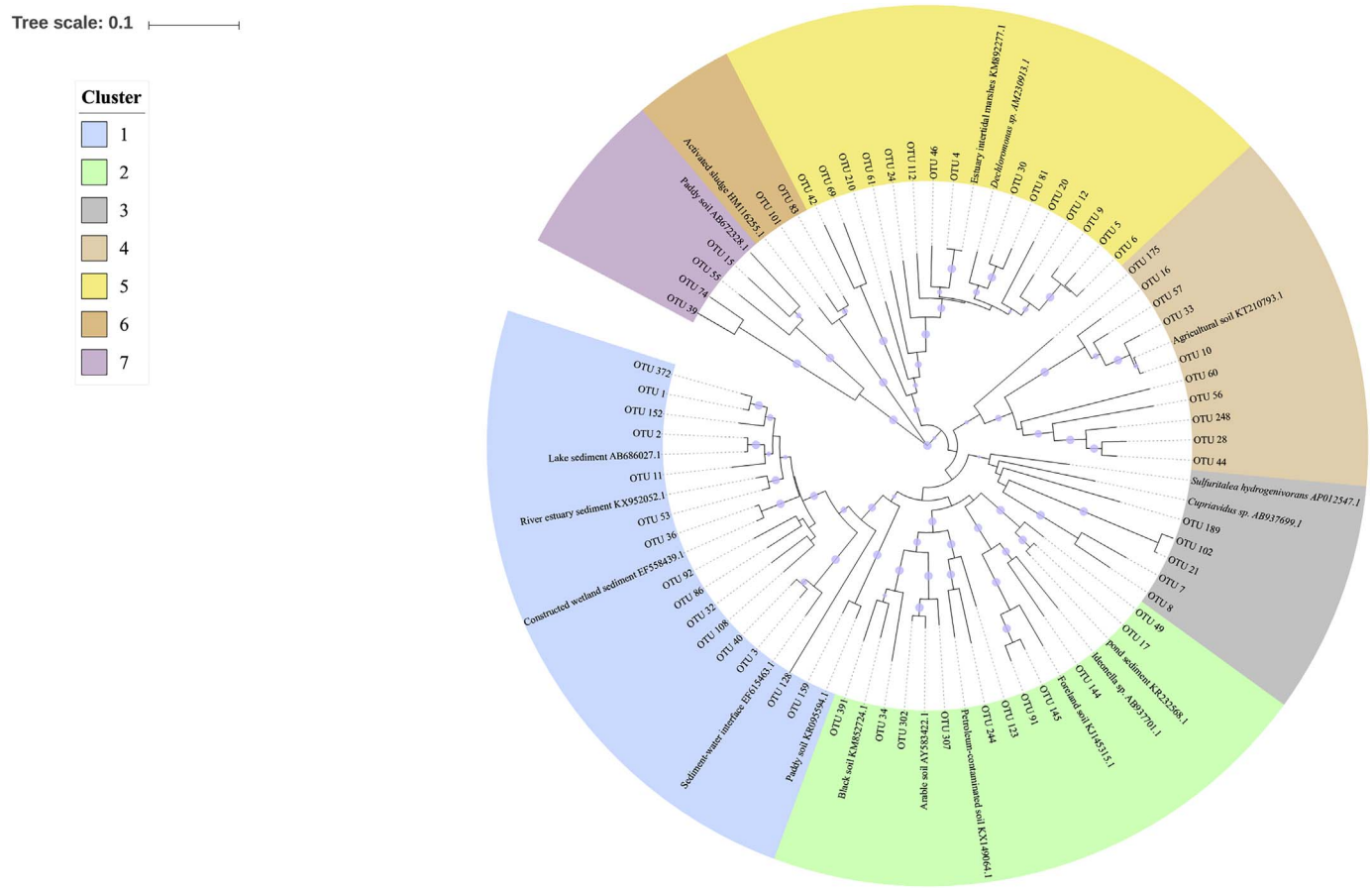

Fig. 5. Phylogenetic analysis of the major *nirS* OTUs (with the relative abundance ≥2% at least in one wetland sediment or soil sample). Branch support values of no less than 50 were dotted. The bar represents 10% sequence divergence based on neighbor-joining analysis.

Table 1
Spearman's rank correlation analysis of wetland sediment or soil environmental factors and denitrification rate with the abundance, richness, and diversity of *nirS*-denitrifier communities.

	NH_4^+-N	NO_3^--N	NO_2^--N	TN	TP	TOC	Temperature	ORP	pH	C/N	Abundance	Chao1 richness	Shannon diversity
Denitrification rate	−0.537[b]	0.558[b]	0.733[b]	0.309	0.458[a]	0.403	−0.358	−0.038	0.661[b]	0.162	−0.045	0.158	0.428[a]
Abundance	0.036	0.074	0.376	−0.178	−0.491[a]	−0.263	−0.587[b]	−0.288	−0.132	−0.352	1.000	0.058	−0.041
Chao1 richness	0.317	0.111	0.371	0.134	0.064	0.257	−0.092	−0.015	0.086	0.180	0.058	1.000	0.660[b]
Shannon diversity	−0.236	0.239	0.425[a]	0.438[a]	0.288	0.708[b]	−0.177	0.090	0.278	0.420[a]	−0.041	0.660[b]	1.000

ORP, TN, TP, TOC and C/N indicate the abbreviations of oxidation and reduction potential, total nitrogen, total phosphorus, total organic carbon, and the ratio of TOC to TN, respectively.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

suggested that riparian wetland denitrification enzyme activity might be related to pH and nitrogen contents (Lan et al., 2015). However, to the authors' knowledge, this was the first report on the potential influence of phosphorus on wetland denitrification rate. In addition, to date, the influence of temperature on wetland denitrification rate remains controversial. Higher denitrification rate during summer than during other seasons was found in an alluvial intertidal wetland (Hu et al., 2014) and a mesocosm-scale CW used for treating reservoir water amended with NH_4NO_3 (Song et al., 2011), whereas Tanaka et al. (2015) reported lower denitrification rate during summer than during spring in a FWSF-CW treating river water. In the present study, sediments and soils in the FWSF-CW treating river water generally had lower denitrification rate during summer than during spring and winter, although Spearman's rank correlation analysis suggested no significant correlation between denitrification rate and wetland sediment/soil temperature.

4.2. Temporal and spatial changes of wetland denitrifier abundance

A few previous studies have revealed spatial or/and temporal dynamics of denitrifier abundance in natural wetlands (Ma et al., 2011; Hu et al., 2014; Gao et al., 2016). The spatial heterogeneity of sediment denitrifier abundance has also been reported in FWSF-CWs treating river water (Ligi et al., 2014; Zhi et al., 2015), yet information about the temporal variation of denitrifier abundance in FWSF-CW treating surface water is still lacking. In this study, *nirS*-denitrifier density in the FWSF-CW treating river water was found to vary among both sampling sites and seasons, ranging from 1.52×10^9 to 1.91×10^{10} gene copies per gram dry sediment/soil, which was also higher than the reported *nirS*-denitrifier density in a coastal wetland (Gao et al., 2016) and a FWSF-CW treating river water (Ligi et al., 2014). In the FWSF-CW, bacterial density varied from 1.71×10^{10} to 1.35×10^{11} 16S rRNA gene copies per gram dry sediment/soil, and the calculated ratio of *nirS*

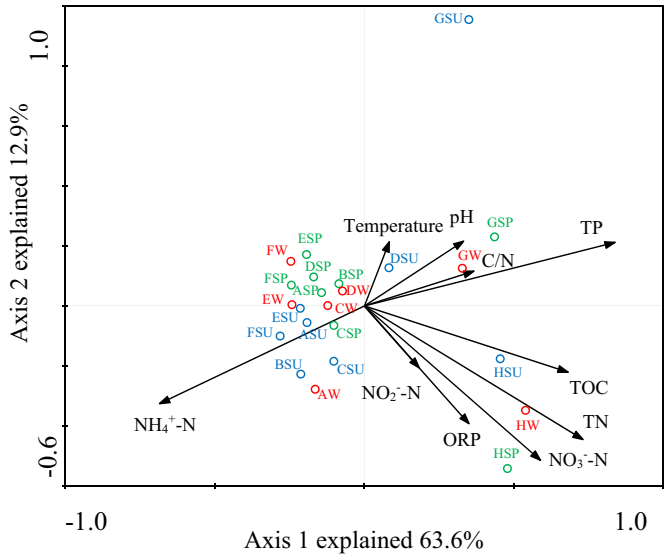

Fig. 6. RDA ordination plot for the first two principal dimensions of the links between *nirS* OTU composition and wetland sediment (or soil) physicochemical parameters.

gene to bacterial 16S rRNA gene was 8.5–23.2% (data not shown). This indicated that *nirS*-denitrifier assemblage was an important component of bacterial community in the studied FWSF-CW. Moreover, the seasonal change pattern of wetland denitrifier abundance was found to be related to plant species. Therefore, vegetation type might have a profound influence on denitrification abundance in the FWSF-CW. The influence of plant type on denitrification abundance has also been documented in a microcosm wetland (Wang et al., 2014).

Several previous studies suggested that denitrifier abundance in sediment/soil of FWSF-CW system might be regulated by water DOC (Zhi et al., 2015) and soil pH (Ligi et al., 2014). TP was found to be a possible determinant of denitrifier abundance in aquic brown soils in Northeast China (Yin et al., 2014). However, the links between phosphorus and wetland sediment/soil denitrifier abundance remain unclear. The present study provided the first evidence for the possible links of wetland denitrifier abundance with sediment/soil TP. Moreover, higher denitrifier abundance during summer than during other seasons was found in a coastal wetland (Gao et al., 2016) and an intertidal wetland (Hu et al., 2014). Denitrifier abundance was even found to be positively correlated with wetland sediment temperature (Gao et al., 2016). In contrast, in the present study, *nirS* gene abundance in the FWSF-CW tended to be lower during summer than during spring and winter. Spearman's rank correlation analysis further confirmed that denitrification abundance in the FWSF-CW might be negatively affected by the increase of sediment/soil temperature.

4.3. Temporal and spatial changes of wetland denitrifier richness and diversity

Direct information about the heterogeneity of wetland denitrifier richness and diversity is still very limited. Based on clone library analysis, Gao et al. (2016) revealed seasonal and spatial variations of *nirS*-denitrifier richness and diversity in a coastal wetland. In this study, high-throughput sequencing was applied to characterize the dynamics of *nirS*-denitrifier richness and diversity in a large FWSF-CW treating river water. The observed values of OTU number, Chao1 richness and Shannon diversity index were 303–758, 398–872 and 4.16–5.18, respectively, which were much higher than those reported in a coastal wetland based on clone library analysis (Gao et al., 2016). Moreover, denitrifier richness and diversity in the studied FWSF-CW varied among both seasons and sampling sites. This was consistent with the previous study (Gao et al., 2016). Gao et al. (2016) revealed no distinctive seasonal shift in wetland denitrifier richness and diversity. The present study further confirmed that the seasonal change pattern of wetland denitrifier richness and diversity differed at sites with different plant species. Hence, plant type might play an important role in determining denitrifier richness and diversity in the FWSF-CW. So far, the environmental factors driving wetland denitrifier richness and diversity remain essentially unclear. Kjellin et al. (2007) suggested that wetland denitrifier diversity increased with decreasing nutrient levels in a FWSF-CW treating wastewater treatment plant effluent. In contrast, in this study, Spearman's rank correlation analysis suggested that denitrifier diversity in the FWSF-CW could increase with the increasing sediment/soil NO_2^--N, TN, TOC and C/N ratio.

4.4. Temporal and spatial changes of wetland denitrifier community structure

A few previous studies have reported remarkable spatial or/and temporal changes of denitrifier community structure in natural wetlands (Angeloni et al., 2006; Baneras et al., 2012; Hu et al., 2014; Gao et al., 2016) and restored wetlands (Peralta et al., 2010, 2012, 2014), yet little is known about the variability of denitrifier community structure in FWSF-CW. Kjellin et al. (2007) revealed the spatial heterogeneity of denitrifier community structure in a FWSF-CW treating wastewater treatment plant effluent, while Song et al. (2012) indicated both seasonal and spatial variations of denitrifier community structure in a FWSF-CW treating river water. In this study, the results of both WPGMA clustering analysis and phylogenetic analysis further confirmed considerable temporal and spatial shifts in denitrifier community structure in the FWSF-CW treating river water. Moreover, the seasonal variation trend for denitrifier community structure differed at sites with different plant species. This suggested the importance of plant type in shaping wetland denitrifier community structure. The influence of plant type on denitrifier community structure had also been reported in a microcosm wetland (Wang et al., 2014), a coastal wetland (Baneras et al., 2012), and an intertidal wetland (Hu et al., 2014).

The links between denitrifier community structure in FWSF-CW and wetland physicochemical features remain poorly known. Only Kjellin et al. (2007) suggested that sediment nitrogen and carbon could affect wetland denitrifier community structure. Moreover, there was a paucity of knowledge on the links of denitrifier community structure with phosphorus in either natural wetland or CW system. In this study, the result of RDA suggested that TP, NH_4^+-N, NO_3^--N, and TN could play important roles in shaping denitrifier community structure in the FWSF-CW. NO_3^--N is used as the substrate for denitrifying microorganisms, and denitrifier community structure illustrated an interactive response to nitrate availability in riparian freshwater wetlands (Morrissey et al., 2013; Morrissey and Franklin, 2015). In addition, sediment NH_4^+-N was also found to be related to denitrifier community structure in a coastal wetland (Gao et al., 2016).

Previous investigations on wetland denitrifier community structure were mainly based on TRFLP (Angeloni et al., 2006; Baneras et al., 2012; Morrissey et al., 2013; Hu et al., 2014; Wang et al., 2014; Li et al., 2015) and DGGE (Kjellin et al., 2007; Tanaka et al., 2015), and phylogenetic information on wetland denitrifier community composition is still very limited. Based on clone library analysis, Gao et al. (2016) revealed that most of *nirS* OTUs from coastal wetlands were closely matched with uncultured environmental *nirS* sequences in the GenBank database, while only a small proportion of *nirS* sequences could be related to the sequence from a cultivated proteobacterial denitrifier. In this study, Illumina MiSeq sequencing indicated that 17.1–46.2% of wetland sediment *nirS* sequences and 0.8–32.3% of soil sequences could be related to those from cultivated denitrifiers. *Dechloromonas*-like denitrifiers were abundant in wetland sediments (13–39.9%) but was a minor group in wetland soils (0.5–4.2%). *Ideonella*-like denitrifiers were detected in each sediment or soil sample, but they only accounted for a small proportion (0.2–8.3%) except for one summer soil sample

(23.3%). In addition, a small proportion of *nirS* sequences (0–10.9%) could be related to those from *Sulfuritalea* and *Cupriavidus* strains.

4.5. Interplay between denitrification rate and denitrifier community in wetland

Denitrification rate was previously found to have a close correlation with denitrifier abundance in natural wetlands (Ma et al., 2011; Hu et al., 2014; Yang et al., 2015). However, in this study, the result of Spearman's rank correlation analysis suggested no direct links between denitrification rate and denitrifier abundance in the FWSF-CW treating river water. Some previous studies indicated that denitrification rate could be affected by denitrifier community structure in natural wetlands (Peralta et al., 2010; Ma et al., 2011; Baneras et al., 2012; Hu et al., 2014) and in a FWSF-CW (Kjellin et al., 2007), while other studies displayed no or poor links of denitrification rate with denitrifier community in FWSF-CWs (Song et al., 2012; Tanaka et al., 2015). In the present study, during each season, wetland soils had much higher denitrification rate than wetland sediments. Denitrifier community structure showed a distinct difference between wetland soils and sediments. Alfalfa and cattail soils also differed greatly in both denitrification rate and denitrifier community structure. Moreover, both denitrification rate and denitrifier community structure in the studied FWSF-CW were regulated by TP, NH_4^+-N and NO_3^--N. These results suggested that denitrification rate might be mediated by denitrifier community structure. There has been no report available on the links between wetland denitrification rate and denitrifier diversity, whereas wetland denitrification rate was found to be positively correlated with denitrifier diversity in the studied FWSF-CW. In addition, the present study provided the evidence for the first time that wetland denitrifier diversity was positively correlated with denitrifier richness.

5. Conclusions

Seasonal and spatial variability in denitrification rate as well as the abundance, richness, diversity and composition of *nirS*-denitrifier community occurred in the FWSF-CW treating river water. Plant type and wetland nitrogen and phosphorus contents could influence both denitrification rate and denitrifier community. Denitrification rate was correlated with denitrifier diversity, instead of abundance. Denitrifier community structure might have a certain influence on denitrification rate.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2017.10.008.

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