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Lower Compositional Variation and Higher Network Complexity of Rhizosphere Bacterial Community in Constructed Wetland Compared to Natural Wetland

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Abstract

Macrophyte rhizosphere microbes, as crucial components of the wetland ecosystem, play an important role in maintaining the function and stability of natural and constructed wetlands. Distinct environmental conditions and management practices between natural and constructed wetlands would affect macrophytes rhizosphere microbial communities and their associated functions. Nevertheless, the understanding of the diversity, composition, and co-occurrence patterns of the rhizosphere bacterial communities in natural and constructed wetlands remains unclear. Here, we used 16S rRNA gene high-throughput sequencing to characterize the bacterial community of the rhizosphere and bulk sediments of macrophyte *Phragmites australis* in representative natural and constructed wetlands. We observed higher alpha diversity of the bacterial community in the constructed wetland than that of the natural wetland. Additionally, the similarity of bacterial community composition between rhizosphere and bulk sediments in the constructed wetland was increased compared to that of the natural wetland. We also found that plants recruit specific taxa with adaptive functions in the rhizosphere of different wetland types. Rhizosphere samples of the natural wetland significantly enriched the functional bacterial groups that mainly related to nutrient cycling and plant-growth-promoting, while those of the constructed wetland-enriched bacterial taxa with potentials for biodegradation. Co-occurrence network analysis showed that the interactions among rhizosphere bacterial taxa in the constructed wetland were more complex than those of the natural wetland. This study broadens our understanding of the distinct selection processes of the macrophytes rhizosphere-associated microbes and the co-occurrence network patterns in different wetland types. Furthermore, our findings emphasize the importance of plant–microbe interactions in wetlands and further suggest *P. australis* rhizosphere enriched diverse functional bacteria that might enhance the wetland performance through biodegradation, nutrient cycling, and supporting plant growth.

Keywords Constructed wetland · Natural wetland · Rhizosphere bacterial community · Co-occurrence network · *Phragmites australis*

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Introduction

Wetlands are transition areas between terrestrial and aquatic ecosystems, which play a vital role in numerous critical ecosystem services, such as biodiversity conservation, water purification, and climate regulation [1]. It is conventionally believed that wetlands can be divided into natural wetlands and constructed wetlands, and there are fundamental differences in the formation and operating conditions between them [2, 3]. Natural wetlands (NWs), the areas that are naturally formed, can function as vital nutrient sinks and pollution buffers through integrated processes involving wetland plants, substrate, and microbial communities [2]. Constructed wetlands (CWs) are artificial ecosystems that utilize

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the same processes occurring in NWs for wastewater treatment and operate in controlled conditions [3, 4]. In many cases, compared to NWs, the capacity for water purification is augmented by CWs due to their specific treatment objectives and targeted operational parameters [5]. Moreover, some management practices in the CWs (e.g., regular plant harvesting and sludge salvage) ensure stable and long-term treatment performance and enhance the efficiency of the treatment system [4]. The wastewater purification of CWs relies on the synergies of physical, chemical, and biological processes [6]. In particular, the biological process mediated by microbes plays a major role in the removal of pollutants in CWs [7]. Furthermore, some vital functional microbes in the rhizosphere have been shown as drivers of the ecosystem functions relevant for biodegradation, thereby would accelerate the purification efficiency of wastewater treatment in CWs [8]. Therefore, investigating microbial communities associated with macrophytes in CWs can broaden our knowledge about the role of microbes in biogeochemical cycling and pollutant degradation, and further provide information for improving the efficiency and sustainability of the CWs.

The macrophyte rhizosphere provides favorable habitat for the growth of microbes by secreting oxygen and exudates [9]. Simultaneously, rhizosphere microbes possess a range of beneficial properties contributing to the productivity and fitness of macrophytes [10, 11]. Numerous studies have well described that macrophytes in NWs could regulate the composition of microbial community and abundance of specific microbial taxa in the rhizosphere to maintain optimal growth [12, 13]. Recently, in CWs, emerging studies have found that the functional microbes enriched in the rhizosphere promote pollutants degradation and nutrient transformation, thus improving the purification efficiency and ecological stability of the wetland ecosystems [14, 15]. For example, Zhao et al. found that the rhizosphere-dominant bacteria such as *Gp6* and *Longilinea* had palpable facilitation in organic pollutants removal, thereby accelerating sewage purification [15]. However, CWs and NWs exhibit significant differences in age, the development of the macrophyte community, environmental properties (e.g., moisture, pH, nutrients, organic matters, etc.), and operational parameters (e.g., hydraulic and pollutant loading, retention time, influent quality, etc.) [5, 16]. Cumulatively, these differences have direct and indirect effects on the rhizosphere recruitment for microbes from surrounding environments. Additionally, the management practices for improving the efficiency of CWs have been reported to lead to spectacular modifications of plant root architecture [17] and exudate composition [18], which presumably affect the establishment of rhizosphere microbial communities. Therefore, we hypothesized that the adaptation of macrophytes to divergent wetland types (i.e., NWs and CWs) could affect the recruitment of rhizosphere from surrounding microbial communities, especially for rhizosphere-enriched microbes which have been proved as key regulators of plant health [19]. Nonetheless, scarce studies have been found in terms of the difference in diversity and composition of the rhizosphere microbial community between these two different wetland types.

In addition to the microbial community composition, interactions within microbes are also a crucial driving force in promoting the growth of plants and a series of vital ecological processes [20]. Co-occurrence networks analysis provides a good tool to decipher the structure of the complex microbial community and predict ecosystem functioning [21]. For instance, Man et al. have found that the complexity of the rhizosphere bacterial community network increased when specific pollutants were added into CWs [22]. The authors suggested that such a response fosters the stability of the rhizosphere bacterial community network and enhances the tolerance of plant host to exogenous pollutants [22]. Similarly, compared to the water column in NWs, water bodies in CWs contain various pollutants from domestic, agricultural, and industrial wastewater, etc. Consequently, the microbial interconnectivity among co-occurring members in CWs likely varies from that of NWs, and such differences may further directly affect purification outcomes. In addition, the network analysis could also identify hub microbial taxa and further indicate their important roles in microbiome structure and functioning [23]. Therefore, uncovering the hub microbial taxa in the microbial community network of different wetlands may help to reveal functional groups responsible for wastewater treatment and better understand the microbially involved pollutants removal mechanisms in the CWs.

Phragmites australis is one of the most commonly found emergent macrophytes in NWs as well as a species commonly employed in the CWs due to its ability to phytoremediation [24, 25]. Therefore, it provides a good framework to investigate the differences in rhizosphere bacterial community in NWs and CWs. In this study, we collected the rhizosphere and bulk sediment samples of *P. australis* in CW and NW nearby the Fuxian Lake, China. We aimed to (i) elucidate the differences in bacterial community composition and diversity between the CW and NW in the rhizosphere of *P. australis*, (ii) identify rhizosphere-enriched bacterial groups and their potential functions in CW and NW, and (iii) analyze the co-occurrence patterns of rhizosphere associated microbes in these two different wetland types.

Materials and Methods

Study Site

Fuxian Lake is one of the largest plateau freshwater lakes as well as the most important conservation areas in China.

The lake, together with its associated NWs, constitute a holistic ecosystem that has being highly valued due to its vital roles in maintaining local ecological balance. However, the water quality deteriorated in the last decades due to high anthropogenic pressure which led to an increase in nutrients and pollutants flowing through the watercourses into the lake. In this scenario, many CWs are designed and widely constructed around the Fuxian Lake to reduce and control the water pollution, therefore providing an ideal research area to explore the macrophyte rhizosphere bacterial community by comparing CWs with the relevant NWs. In the present study, two wetlands nearby the Fuxian Lake, Yunnan Province, China were selected (Fig. S1). The first one is an unmanaged endorheic wetland of natural origins in the south of the Fuxian Lake (24°24′17″ N, 102°50′3″ E), with *P. australis* as the dominant species and the water from the Fuxian Lake as the main water source. The second one is a CW named "Yaonigou" that has been built in the north of the Fuxian Lake (24°37′56″ N, 102°54′26″ E). During the operation period, Yaonigou CW showed its advantages such as low investment and running cost, high efficiency, and beautiful sightseeing, which provides an example for the treatment of agricultural and domestic wastewater. Additionally, these two wetlands were chosen to compare the rhizosphere bacterial communities of *P. australis* between NW and CW because of their partially overlapping in terms of vegetation type and water source.

Yaonigou CW was constructed in 2001. This wetland system is mainly used for the treatment of the domestic and agricultural wastewater from the Yaonigou ditch, and an agricultural irrigation ditch, so as to reduce the pollutant loading being discharged to the Fuxian Lake. With an effective area of 2.2 hm^2, Yaonigou CW is comprised of two projects, Project I and Project II, which operated independently and have a similar design of the wetland system. The present study focused on the Project I considering its higher hydraulic loading of 3000 m^3/day compared to the Project II. The hydraulic retention time of the studied wetland system is ~ 4 days and eventually, the purified water is discharged to the Fuxian Lake. The sewage treatment process was described as follows: inflow → precipitation tank → biological oxidation pond → aeration tank → subsurface flow wetland → surface flow wetland → outflow. The subsurface flow wetland and surface flow wetland, the heart of the studied wetland, vegetated with *Canna indica L.* and *P. australis*, respectively. The removal efficiencies of Yaonigou CW for total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD), and suspended solids (SS) were 52.8%, 56.4%, 61.4%, and 90.7%, respectively. The management practices of this system include aquatic plant harvesting in the winter and sludge salvage annually.

Field Sampling

Rhizosphere and bulk sediment samples were collected in the peak vegetation growth stage (August) of *P. australis* in 2018. The sampling site in NW with *P. australis* as the dominant species and surface flow wetland unit planted with *P. australis* in CW were selected. To avoid the bias caused by random environmental variation, we selected two 10-m by 10-m plots for sampling in each wetland. Within each plot, samples were collected at four vertex positions. Eight sediment cores at a 10 cm diameter and 5–15 cm depth were collected, with each sediment core containing a root of individual *P. australis*. All the sediment cores containing root tissue were taken back to the laboratory for further processing. Eight bulk sediment samples without any plant tissue around the vegetate were obtained adjacent to the excavated plant (30 cm from the vegetated area) at the same depth using a core sampler (DM60, Mingyu, China) and mixed manually. In total, 32 sediment samples (eight rhizosphere and eight bulk sediment samples of each type of wetland) were collected and then kept at 4 ℃, and then transported to the nearest cooperative laboratory within 2 h of collection.

All the bulk and root sediment samples were lyophilized by a vacuum freeze dryer (LABCONCO, USA). Rhizosphere samples were separated from the root zones according to İnceoğlu et al. [26]. Briefly, we first removed the sediment that loosely adhered to the root by shaking off the sediment core, and then a rhizosphere sample was collected by brushing off the sediment that was tightly attached (< 1 mm) to the root surface using a sterile spoon. Bulk and rhizosphere sediment samples for physicochemical and DNA analysis were ground and homogenized and then sieved to 0.5 mm. All samples were stored at – 80 ℃.

Sediment Physicochemical Properties Analysis

Physicochemical parameters of all the sediment samples containing total phosphorous (TP), total nitrogen (TN), pH, and loss on ignition (LOI) were examined according to Zeng et al. [27]. The significant difference of each physicochemical property between two sediment compartments and two wetland types was assessed through the nonparametric Mann–Whitney *U* test in SPSS (v.23.0) software (IBM-SPSS, Chicago, Illinois, USA).

DNA Extraction, Polymerase Chain Reaction Amplification, and High-Throughput Sequencing

Total bacterial DNA of each bulk and rhizosphere sediment sample was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA). Detailed protocols for DNA purification and quantification have been described previously [27]. Each sample was extracted for

three times to reduce bias during the DNA extraction process. And then the triplicate DNAs from each sample were mixed and kept at – 80 ℃ before further processing.

The hypervariable region V4 of the bacterial 16S rRNA gene was amplified using the primer sets 515F (5′-GTG CCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTA CHVGGGTWTCTAAT-3′). The PCR amplification was conducted using a consistent protocol with our previous study [28]. Triplicate PCR amplification products were pooled together after verifying with 2% (wt/vol) agarose gel electrophoresis. The combined PCR products were purified with the AxyPrepDNA gel purification kit (Axygen Biosciences, Union City, CA, USA) and quantified using Qubit®3.0 (Life Invitrogen). High-throughput sequencing was performed at an Illumina MiSeq platform (Novogene Co., Ltd., Beijing, China). The obtained raw data in this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession Number: PRJNA605303).

Sequence Data Processing

We first demultiplexed raw data into the corresponding sample according to the index sequences generated from sequencing and then pre-processed with QIIME (v1.9.1) [29]. Pair-end 250 bp reads with quality (quality score < 25) or sequence length < 10 bp were trimmed by Trimmomatic (v.0.33) [30]. Only the truncated reads having an overlap length > 10 bp and a mismatch < 0.25 were reserved for merging using FLASH (v.1.2.11) [31]. Subsequently, chimeric sequences were identified and discarded using the USEARCH tool (v.4.2.52) based on the UCHIME algorithm [32]. By running the command "*pick_rep_set.py*" in QIIME, we clustered high-quality sequences into operational taxonomic units (OTU) at a 97% similarity. Representative sequences of each OTU were obtained by aligning against the RDPClassifier_16S_trainsetNo16_QiimeFormat database [33]. Taxonomic classification based on the online tool RDP (Ribosomal Database Project) classifier at a bootstrap cutoff of 80% [33]. Low-frequency (< 0.0005% of the total sequence) and Archaea, Chloroplast, Mitochondria, and unclassified were removed. The aligned and filtered sequences were applied to calculate the phylogenetic tree in FastTree (v.2.1) [34]. Finally, we obtained 2,928,023 high-quality sequences from 32 sediment samples and 12,626 operational taxonomic units (OTUs) at the 97% sequence similarity level. The relative abundance of each OTU at different taxonomic levels was calculated in QIIME. After that, a normalized OTU BIOM table aiming to avoid biases associated with different sequencing depths was generated for alpha and beta diversity analysis by randomly rarefying all samples to the lowest sequence number (58,404 sequences).

Bacterial Community Diversity and Composition Analysis

Bacterial community alpha diversity indices represented by OTU richness, Shannon diversity index, and Faith's phylogenetic diversity index were calculated using the command "*alpha_diversity.py*" in QIIME. The significant differences in alpha diversity indices between two sediment compartments and two wetland types were assessed through the nonparametric Mann–Whitney *U* test. Meanwhile, Spearman's correlations between environmental factors and each alpha diversity index were conducted in SPSS (v.23.0). The linear and quadratic regression models were selected based on a lower value of the Akaike information criterion (AIC) [35] using the commands "*lm*" and "*AIC*" in R. Bacterial community beta diversity was calculated based on Bray–Curtis dissimilarity distance matrix using the command "*beta_diversity.py*" in QIIME. Principal coordinate analysis (PCoA) was used to visualize the compositional differences of bacterial communities, and permutational multivariate analysis of variance (PERMANOVA) was used to quantify and test the significant influence of sediment compartment and wetland type on variance within the bacterial community. Both PCoA and PERMANOVA were conducted with the "*vegan*" package in R (v.3.6.3). The relative abundance of the dominant phylum (> 1%) was examined for the difference between different sediment compartments and wetland types via Mann–Whitney *U* test. To identify rhizosphere-enriched bacterial taxa in NW and CW, we first determined the unique and shared OTUs between bulk and rhizosphere sediment samples in NW and CW, respectively, and graphically represented in a Venn diagram using the "*VennDiagram*" package. Next, the rhizosphere-enriched OTUs within each wetland type were identified by applying linear discriminant analysis effect size (LEfSe) (Wilcoxon rank-sum test, $P < 0.05$, logarithmic LDA (linear discriminant analysis) score > 2) among the shared OTUs. Specifically, OTUs significantly abundant in rhizosphere samples were grouped as rhizosphere-enriched OTUs, while OTUs significantly abundant in bulk samples were grouped as bulk-enriched OTUs, and those with no significant differences in relative abundance between bulk and rhizosphere samples were grouped as "Others". The relative abundances of the top 10 rhizosphere-enriched OTUs of each wetland type were presented in a heat map using the "*pheatmap*" package.

Co-occurrence Network Construction

In this study, co-occurrence network analyses were performed using Sparse Correlations for Compositional data (SparCC) to provide insight into the structure and putative ecological interactions among bacterial taxa [36]. We

constructed four networks for bulk and rhizosphere bacterial communities corresponding to NW and CW, respectively. In practice, we provisioned 8 samples for each group and kept OTUs that occurred in more than 50% and had relative abundance greater than 0.01% to network construction. Only strong (SparCC $|R|>0.9$) and statistically significant (P value <0.001) correlations were accepted in the co-occurrence networks. The nodes in the networks represent the OTUs at 97% similarity and edges represent strong and significant associations between OTUs. All networks were visualized with the interactive platform Gephi (v.0.9.2) [37]. While, 1000 random networks with consistent nodes and edges were randomly generated for each group. Network topological attributes including the numbers of network nodes and edges, modularity, clustering coefficient, average degree path, network diameter, average degree, and graph density were calculated for both empirical and random networks using the "*igraph*" package in R. The significant differences of these topological attributes between empirical and random networks were tested by using *Z*-test.

A bacterial community network with the modularity value >0.4 is believed to be modular, and the functionality of a complex network largely depends on its modular degree [38]. Consequently, we determined the role of the node structuring the networks using two parameters: within-module connectivity (Z_i) (describe how the node is positioned within a specific module) and among-module connectivity (P_i) (describe how the node interacts with other modules) according to Guimera and Amaral [39]. Here, nodes in a modularized network were classified as peripherals nodes ($Z_i \leq 2.5$, $P_i \leq 0.62$), module hubs ($Z_i > 2.5$, $P_i \leq 0.62$), connectors ($Z_i \leq 2.5$, $P_i > 0.62$), and network hubs ($Z_i > 2.5$, $P_i > 0.62$). The peripheral nodes were nodes that have few links with other nodes, the module hubs were nodes that were highly connected within modules, the connectors were nodes that provide links among several modules, and network hubs were nodes that were highly connected both within and among modules.

Results

Physicochemical Characteristics of Sediments

The physicochemical properties of bulk and rhizosphere sediments varied in different wetlands (Fig. S2). Briefly, bulk and rhizosphere sediment samples collected in CW both presented higher LOI, TN, and TP than those sampled from NW (Mann–Whitney U test, $P<0.001$). Whereas the rhizosphere sediments of NW had higher pH compared to that of CW (Mann–Whitney U test, $P<0.01$). Additionally, LOI and TN showed a significantly increasing value from the bulk to rhizosphere sediments in CW (Mann–Whitney U test, $P<0.05$). Meanwhile, an increase in TN was observed from the bulk to rhizosphere sediments in NW (Mann–Whitney U test, $P<0.05$).

Diversity and Composition of Bacterial Community

The rarefaction curves for the bacterial communities reached approximate saturation, suggesting that the sequencing depth was sufficient for all samples in the present study (Fig. S3). We determined bacterial community alpha diversity based on OTU richness, Shannon diversity index, and Faith phylogenetic diversity index. In the NW, all bacterial community alpha diversity indices were significantly higher for the rhizosphere than those for the bulk sediment samples (Mann–Whitney U test, $P<0.05$) (Fig. 1). However, this trend was not observed in the CW, in which bulk and rhizosphere bacterial communities were equally diverse (Mann–Whitney U test, $P>0.05$) (Fig. 1). Alternatively, bulk and rhizosphere bacterial communities from CW both showed a greater value than those from NW for all alpha diversity indices (Mann–Whitney U test, $P<0.05$) (Fig. 1). The alpha diversity indices of the bacterial community showed significantly positive linear relationships with LOI and TP as well as significantly positive quadratic relationships with TN (Fig. S4). Spearman's correlations analysis also demonstrated that environmental factors including LOI, TN, and TP exhibited strong positive correlations with all the alpha diversity indices (Table S1).

Regarding the bacterial community beta diversity, PCoA and PERMANOVA based on the Bray–Curtis distance matrix were used to visualize and quantify the differences in the bacterial community. Wetland type alone explained the largest overall variability in the bacterial community composition ($R^2=0.467$, $P<0.001$, PERMANOVA; Table 1). Consequently, the samples were clearly separated by wetland type along the first principal coordinate (Fig. 2a). Additionally, the comparison between the different wetland types revealed that the rhizosphere bacterial communities were significantly more similar than bulk bacterial communities (Fig. 2b). The sediment compartment was the second-largest source of variation within the bacterial community ($R^2=0.138$, $P<0.001$, PERMANOVA; Table 1), with distinctly different and separately clustered bacterial communities between the rhizosphere and bulk sediments (Fig. 2a). Although bacterial communities within each wetland were significantly affected by the sediment compartment, the effect was more pronounced for NW than for CW considering the more similar bacterial community composition between rhizosphere and bulk sediment samples from CW (Fig. 2c). While, we also noticed the significant cooperatively influence of wetland type and sediment compartment on bacterial community composition ($R^2=0.112$, $P<0.001$, PERMANOVA; Table 1).

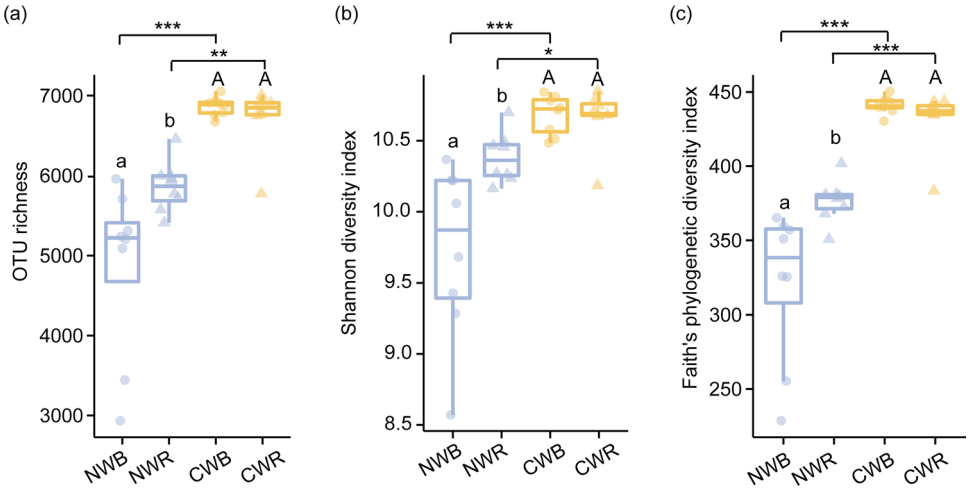

Fig. 1 Comparative analysis of alpha diversity of bulk and rhizosphere bacterial communities in natural and constructed wetlands. **a** OTU richness. **b** Shannon diversity index. **c** Faith's phylogenetic diversity index. Lowercase and uppercase letters above boxes denote statistically significant differences (Mann–Whitney *U* test, $P < 0.05$) of bacterial community diversity between the bulk and rhizosphere sediments in natural and constructed wetlands, respectively. Asterisks indicate significant differences between two wetland types in the same compartment (Mann–Whitney *U* test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). *NWB* natural wetland bulk, *NWR* natural wetland rhizosphere, *CWB* constructed wetland bulk, *CWR* constructed wetland rhizosphere

Table 1 Permutational multivariate analysis of variance (PERMANOVA) results with Bray–Curtis distance matrix revealing the sources (wetland type, compartment, interaction between wetland type and compartment) of variation in bacterial communities. Statistical significance conducted based on sequential sums of squares from 999 permutations

Source of variation	Df	SS	MS	R^2	Pseudo-*F*	*P*
Wetland type	1	2.392	2.391	0.467	46.172	0.001
compartment	1	0.705	0.705	0.138	13.604	0.001
Interaction	1	0.574	0.574	0.112	11.088	0.001
Residuals	28	1.450	0.052	0.283		
Total	31	5.121		1		

Df degrees of freedom, *SS* sums of squares, *MS* mean squares, *Pseudo-F F*-model, *P* statistical significance computed based on sequential sums of squares from 999 permutations

Dominant bacterial phylum and classes for *Proteobacteria* with relative abundance greater than 1% were shown in Fig. S5. In bulk bacterial communities, *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes* had greater abundance in NW than that in CW, whereas *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia* were more abundant in CW compared to NW. In rhizosphere bacterial communities, NW had a significantly higher relative abundance of *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia*, but a lower relative abundance of phylum *Chloroflexi*. Interestingly, NW rhizosphere sediment was enriched in *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Verrucomicrobia*, while depleted in *Chloroflexi* and *Firmicutes* compared to bulk sediment. However, for bacteria in CW sediment, no bacterial phyla were found to exhibit substantial differences in the relative abundance between bulk and rhizosphere sediment samples.

Rhizosphere-Enriched Bacterial Community in Natural and Constructed Wetlands

We further performed a comparison of the rhizosphere-enriched bacterial community at the OTU level between different wetland types. In total, we found 451 and 27 rhizosphere-enriched OTUs for the NW and CW, respectively, among them, 20 OTUs were shared between different wetland types (Fig. 3a). Meanwhile, these rhizosphere-enriched OTUs accounted for 46.84% and 4.35% of the total rhizosphere bacterial community sequences in NW and CW, respectively. Additionally, it should be noted that 428 out of 451 rhizosphere-enriched OTUs in NW were also present in the *P. australis* rhizosphere sediment of CW, and the 27 rhizosphere-enriched OTUs in CW were all present in

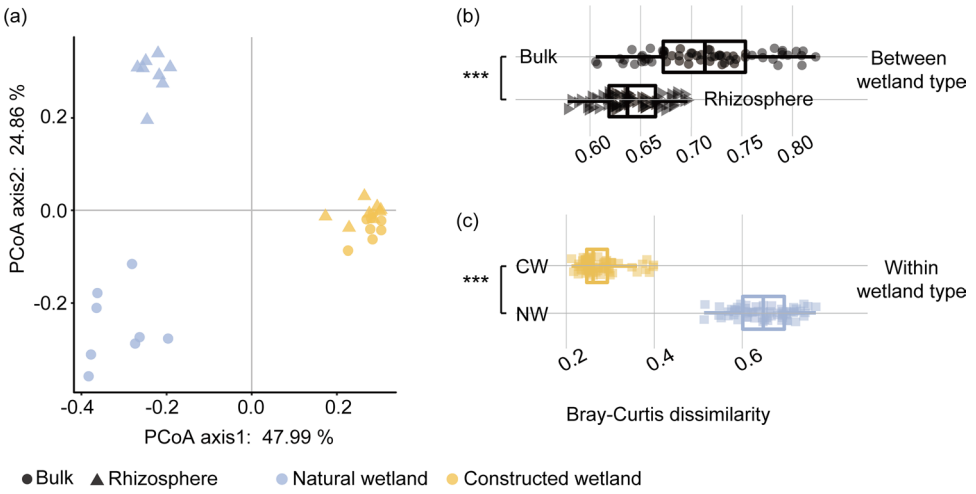

Fig. 2 Bacterial community beta diversity patterns. **a** Principal coordinate analysis (PCoA) of bulk and rhizosphere bacterial communities in natural and constructed wetlands based on the Bray–Curtis distance matrix. Samples are color-coded and shape-coded based on wetland type and sediment compartment, respectively. **b** Distribution of pairwise Bray–Curtis dissimilarities between wetland type in the bulk and rhizosphere bacterial communities. **c** Distribution of pairwise Bray–Curtis dissimilarities between bulk and rhizosphere bacterial communities within each wetland type. In both b and c, asterisks suggest significant differences (Mann–Whitney *U* test, $P < 0.001$). *NWB* natural wetland bulk, *NWR* natural wetland rhizosphere, *CWB* constructed wetland bulk, *CWR* constructed wetland rhizosphere

the *P. australis* rhizosphere sediment of NW. The relative abundance of the top 10 rhizosphere-enriched OTUs with annotations of the minimum taxonomic level was listed in a heat map (Fig. 3b). The relative abundance of four genera including *Ramlibacter*, *Dechloromonas*, *Hydrogenophaga*, *Piscinibacter*, and one family *Rhodocyclaceae* (all affiliated with *Betaproteobacteria*) were consistently enriched in NW rhizosphere sediment (Fig. 3b). Genera including *Pseudomonas* (affiliated with *Gammaproteobacteria*) and *Rhodospirillum* (affiliated with *Alphaproteobacteria*) were also preferably enriched in NW rhizosphere sediment (Fig. 3b). In contrast, some genera such as *Rhodoferax*, *Polaromonas*, *Ferribacterium* (all affiliated with *Betaproteobacteria*), *Desulfobulbus*, *Desulfocapsa* (both affiliated with *Deltaproteobacteria*), *Methylocaldum* (affiliated with *Gammaproteobacteria*), and *Flavobacterium* (affiliated with *Bacteroidetes*) were abundant exclusively in CW rhizosphere sediment (Fig. 3b).

Contrast Network Complexity of Bacterial Community Between Natural and Constructed Wetlands

To investigate the co-occurrence patterns of bacterial communities, we established bacterial community co-occurrence networks and then calculated the network topological features (Fig. 4; Table 2). The bacterial community network in bulk sediment of NW was characterized by more nodes and edges, higher average degree, higher average path length, and larger network diameter than those of bulk bacterial community network in CW. These suggested a larger and more interconnected structure for bulk bacterial community network in NW (Table 2). Contrastingly, this trend was reversed in the rhizosphere bacterial community networks. Rhizosphere bacterial community in NW formed a larger but less interconnected network relative to that of CW, as revealed by fewer nodes and edges, lower average degree, higher average path length, and larger network diameter in the bacterial community network of the rhizosphere in NW compared to those of CW (Table 2). Additionally, the rhizosphere bacterial community networks differed profoundly from the bulk bacterial community networks, and these differences varied between NW and CW. The NW rhizosphere bacterial community network contained fewer nodes and edges but higher modularity than those of bulk bacterial community network. While in CW, the rhizosphere bacterial community network was more connected and complex, and less modular than the bulk bacterial community network. Meanwhile, we found rhizosphere bacterial community networks, especially for CW, consistently had more negative links than bulk bacterial community networks (14.81% versus 12.43% and 43.87% versus 14.29% for rhizosphere and bulk bacterial community networks in NW and CW, respectively). We also mapped the enriched OTUs in each compartment (as identified in Fig. 3a) into the co-occurrence networks. We found the enriched OTUs, especially in bacterial community networks of NW (accounting for 47.71% and 58.76% of the number of total nodes for bulk and rhizosphere bacterial community networks, respectively), exhibited high connectivity with other OTUs and overwhelmingly

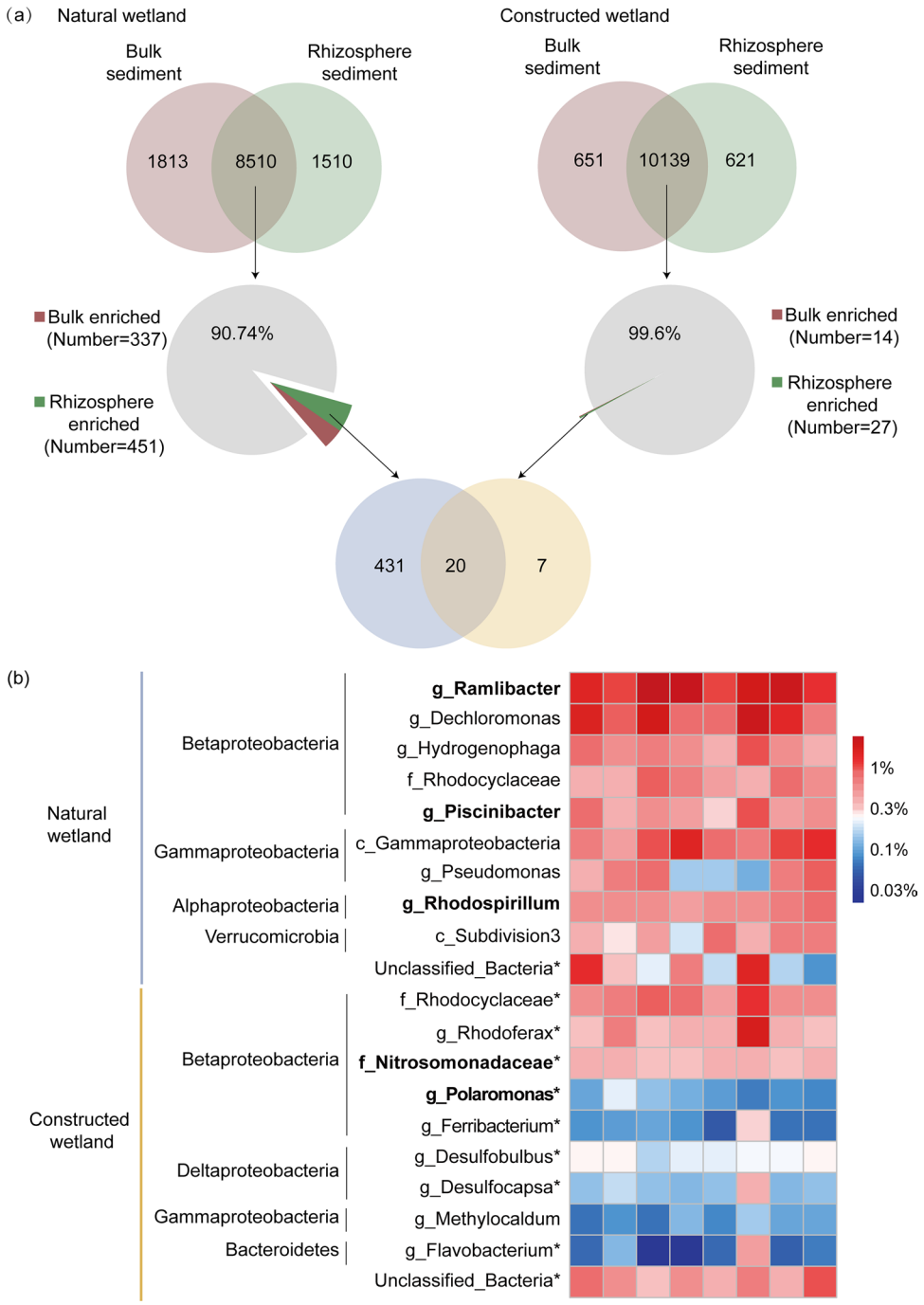

Fig. 3 Rhizosphere-enriched OTUs in natural and constructed wetlands. **a** Numbers of unique and shared OTUs between bulk and rhizosphere sediments in natural and constructed wetlands, respectively. From the shared OTUs, we identified bulk- and rhizosphere-enriched OTUs within each wetland type based on LEfSe with an alpha value of 0.05 and a threshold of 2. **b** Heatmap showing the top 10 abundant rhizosphere-enriched OTUs within natural and constructed wetlands. Asterisks indicate the OTU shared by rhizosphere-enriched OTUs of natural and constructed wetlands. Bold indicates taxonomic information of these OTUs was obtained based on alignment against the NCBI database using the BLAST algorithm. The color bar indicates the relative abundance (%) of each OTUs

positive interactions within each other (Fig. 4). Nevertheless, the number of enriched OTUs in bacterial community networks of CW networks was relatively lower, with six and ten enriched OTUs for bulk and rhizosphere bacterial community networks, corresponding to 6.52% and 4.33% of the number of total nodes, respectively. Specific taxonomic information of enriched-OTUs in each bacterial community network was shown in Table S2.

Topologically important OTUs were identified in the co-occurrence network based on their among-module connectivity (P_i) values and within-module connectivity (Z_i) (Fig. S6). The majority of the nodes in each bacterial community network exhibited a common feature: most of their links were inside their modules. No network hubs were identified in all bacterial community networks as well as no module hubs or connectors were identified in the bulk bacterial community network in CW. Considering the potential ecological role of hub bacterial taxa, we examined whether any of these bacterial taxa also appeared as enriched taxa. Two module hubs (families

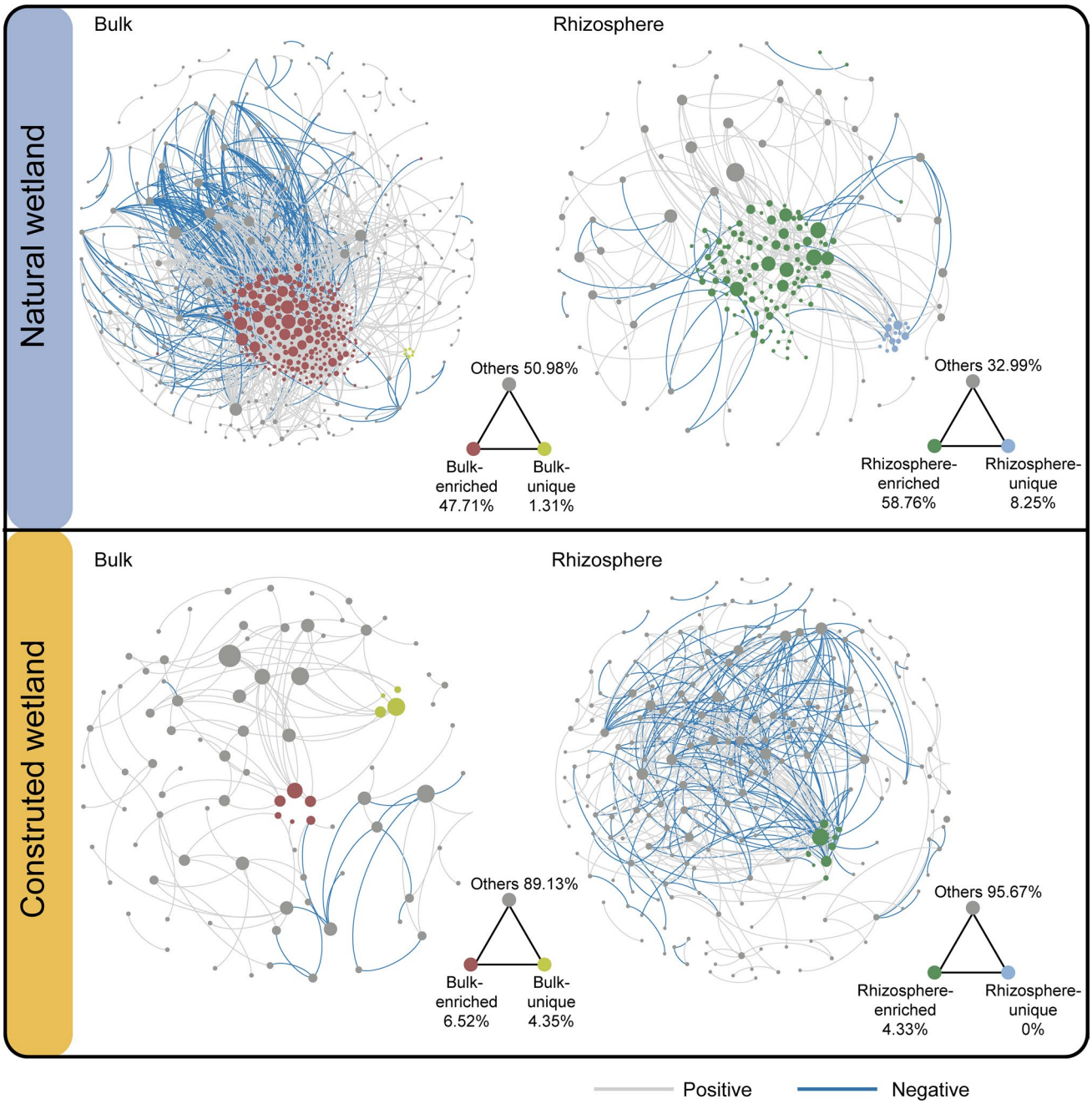

Fig. 4 Co-occurrence networks for bulk and rhizosphere bacterial communities in natural and constructed wetlands. Each node represents an OTU, and the size of each node is proportional to the degree of the OTUs. The nodes (OTUs) were colored based on compartment-enriched/unique for natural and constructed wetlands, respectively. Each edge connecting two nodes indicates the relationship between these two OTUs, and the color of each edge means positive/negative correlation

Anaerolineaceae and *Micromonosporaceae*) and nine connectors (members included orders *Gp10* and *Myxococcales*, class *Subdivision3*, family *Anaerolineaceae*, as well as genus *Gemmatimonas* and *Terrisporobacter*, and three bacterial taxa that could not be identified at the phylum level) of the bulk bacterial community network in NW were identified as enriched OTUs (Table S3). Two OTUs were classified as module hubs of the rhizosphere bacterial community network in NW, including OTUs affiliated with order *Myxococcales* and class *Gammaproteobacteria* (Table S3). Hubs of rhizosphere bacterial community network in CW that were also identified as enriched OTUs included members order *Bacteroidales*, family *Rhodocyclaceae*, and genus *Rhodoferax* (Table S3).

Table 2 Topological properties of the empirical and random bacterial community co-occurrence networks

Empirical network								Random network			
Type	Nodes	Edges	Modularity	Clustering coefficient	Average path length	Network diameter	Average degree	Graph density	Modularity (SD)	Clustering coefficient (SD)	Average path length (SD)	Network diameter (SD)
NWB	457	Positive 1267	0.526[a]	0.407[a]	5.010[a]	20[a]	6.775	0.015	0.356 (0.005)	0.015 (0.002)	3.412 (0.007)	6.292 (0.474)
		Negative 281										
NWR	194	Positive 200	0.871[a]	0.316[a]	6.209[a]	16[a]	2.454	0.013	0.658 (0.015)	0.013 (0.009)	5.503 (0.223)	12.946 (1.398)
		Negative 38										
CWB	92	Positive 98	0.796[a]	0.312[a]	2.700[a]	6[a]	2.478	0.027	0.607 (0.021)	0.026 (0.019)	4.609 (0.248)	10.672 (1.248)
		Negative 16										
CWR	231	Positive 277	0.601[a]	0.375[a]	4.783[a]	15[a]	4.216	0.018	0.475 (0.009)	0.018 (0.006)	3.896 (0.038)	8.162 (0.625)
		Negative 210										

NWB natural wetland bulk, *NWR* natural wetland rhizosphere, *CWB* constructed wetland bulk, *CWR* constructed wetland rhizosphere

[a]Significant difference ($P < 0.05$) between empirical network and random network based on *Z*-test

Discussion

Comparison of Rhizosphere Bacterial Community Composition and Diversity Between Natural and Constructed Wetlands

Our results revealed the significant differences in bacterial community composition and diversity between CW and NW in the rhizosphere of *P. australis* (Fig. 1; Fig. 2). Meanwhile, consistent with previous studies, distinct bulk bacterial communities were also found between CW and NW [2, 40]. These differences observed in bacterial communities could be attributed to the differential sedimental physicochemical properties between CW and NW shift bacterial communities. In the present study, strong influences of wetland type on sediment physicochemical properties were visible in the higher TN, TP, and LOI in the CW compared to those in NW (Fig. S2). Therefore, wetland type effects on sediment properties such as nutrient level and organic matter likely contribute greatly to bacterial community assembly. Intriguingly, we found that the similarity of bacterial community composition between NW and CW in the rhizosphere (NWR vs CWR) was greater than that in bulk (NWB vs CWB) (Fig. 2b). This result suggested that plants are also important drivers of the changes in rhizosphere microbes as well as consistently imposed a strong selective filter [41]. Nevertheless, the magnitude of plant selective effects on the composition of rhizosphere bacterial community differed between NW and CW. As we found fewer differences in bacterial community composition between bulk and rhizosphere in CW compared to NW (Fig. 2c). Compared to an oligotrophic bulk sediment compartment, rhizosphere oxygen secretion and root exudates together provide an oxygen-enriched and nutrient-available sediment microhabitat for bacteria, likely favoring the divergence of bulk and rhizosphere bacterial communities [42, 43]. Since the higher nutrient (in terms of TN and TP) and organic matter (in terms of LOI) input in the CW (Fig. S2), the nutrient-level-induced changes in bacterial community composition were likely similar in the bulk and rhizosphere sediments. Whereas plants growing in the NWs might have encountered a nutrient-enriched root zone already distinct from the bulk sediment. Furthermore, the direction of the plant selective effects also varied with wetland types for rhizosphere bacterial community composition, as revealed by distinct rhizosphere bacterial communities between NW and CW (Fig. 2a). The plant adaption to engineered systems may mediate some enrichment of specific bacteria in the rhizosphere, as contaminated water bodies and some management practices in CW may trigger active responses of related bacteria to complex molecular and physiological processes [44, 45].

In addition, we found higher bacterial community alpha diversity of sediment samples in CW compared to those of NW, which was in agreement with the previous study [46]. Zhou et al. have suggested that nutrient input favored the maintenance of higher bacterial community alpha diversity by weakening resource competition [47]. This explanation may apply here as well, specifically with regards to the excess nutrient input in CW. And we also found that sediment bacterial community alpha diversity indices have a significantly positive correlation with LOI, TN, TP (Fig. S4; Table S1), suggesting the higher nutrients facilitates the colonization of bacteria in CW.

Rhizosphere Bacterial Community Enrichment Patterns

Rhizosphere-enriched bacterial taxa were identified in both NW and CW (Fig. 3). We found that a considerable number of OTUs were only enriched in the rhizosphere of either NW or CW. A central question is whether the observed rhizosphere-enriched bacterial community is beneficial to the host plants. Therefore, we inspected these rhizosphere-enriched OTUs for taxa with known potential functions that are of importance for plant adaptation to system-specific environmental conditions. We found several well-described nitrifiers or denitrifiers with potential functional importance, including *Ramlibacter* [48], *Dechloromonas* [49], *Hydrogenophaga* [50], and *Piscinibacter* [51] were exclusively enriched in the rhizosphere of NW. In addition, *Pseudomonas*, a genus that has been reported as important plant growth-promoting rhizobacteria (PGPR) [52], was also exclusively enriched in the rhizosphere of NW. Some strains of this genus in the rhizosphere could greatly promote plant adaptation to diverse environmental conditions by various mechanisms, such as Indole-3-acetic acid (IAA) and cytokinin production [53]. In CW, a majority of rhizosphere-enriched OTUs have been isolated and characterized in previous studies, and have shown exceptional biodegradation potential for a variety of contaminants from various environments, including *Rhodoferax* (sulfolane-degrading) [54], *Polaromonas* (toluene-degrading) [55], *Ferribacterium* (benzene-degrading) [56], and *Flavobacterium* (dioxane-degrading) [57]. Additionally, *Desulfobulbus* and *Desulfocapsa* were both enriched in the rhizosphere of CW; these two taxa have been suggested to be involved in sulfate reduction [58, 59]. Moreover, *Methylocaldum* is thermophilic and methanotrophic; this genus was also enriched in the plant rhizosphere of CW [60]. Hence, the differential enrichment of specific bacterial taxa in the rhizosphere between NW and CW might be a plant-regulated mechanism to maintain active microbes in the face of divergent environmental conditions through enrichment of specific taxa with adaptive functions. Meanwhile, the enrichment of those OTUs in the rhizosphere of CW properly suggested the application potential of *P. australis* in CW by enriching bacteria that participate in biodegradation and nutrient cycling. However, we only sampled in one NW and one CW nearby the Fuxian Lake, which offers a possibility that the identified differences in situ in the enrichment patterns of the rhizosphere bacterial community between NW and CW in the present study may be limited to our sample sites. There remain uncertainties that whether the selective enrichment patterns of rhizosphere bacterial community were wetland type-dependent; therefore, further investigations including a series of relevant CWs and NWs are required.

Bacterial Community Co-occurrence Patterns

In this study, network analysis revealed the bacterial community network of NW bulk sediment consisted of the most nodes and edges, whereas the bacterial community network of CW bulk sediment comprised of the least nodes and edges (Table 2). One possible explanation for the differential complexity in terms of the bulk bacterial community network is the high nutrient input in CW. Greater resource availability usually reduces competition within bacterial communities [61]. Consequently, it would be expected that the bulk bacterial community network of NW was more complex than that of CW due to stronger competition for resources. Additionally, network properties may be also dramatically affected by the differential operational parameters between CW and NW. For example, the previous study has suggested that the hydraulic retention time greatly affected the bacterial interactions of the anammox process [62]. However, we found higher bacterial community network complexity of rhizosphere in CW than that in NW. Specifically, we observed higher average degree and clustering coefficient but lower average path length and network diameter of rhizosphere bacterial community network in CW compared to those of the NW (Table 2), indicating nodes of rhizosphere bacterial community network in CW were more clustered and closely connected with plenty edges. Wastewater is characterized by high levels of nutrients, as well as pollutants and toxic matters, which may cause harsh abiotic conditions for the growth of plants [63]. Hassani et al. have demonstrated that the enhanced interactions within the rhizosphere bacterial community may alter plant growth and fitness in beneficial ways [64]. Therefore, the increased associations within the rhizosphere bacterial community of CW may be the positive feedback of plant–microbe to environmental stress. For example, de Vries et al. have suggested that the increase of negative interactions among bacteria is helpful to reinforce the resilience of bacterial communities to disturbances, and thus potentially fosters plant growth [65]. Similarly, our results also revealed a higher proportion of

negative links in the rhizosphere bacterial community network of CW (43.87%) than that of NW (14.81%) (Table 2), which may improve the tolerance of rhizosphere bacterial communities to external pressures, and subsequently promote plant performance [66]. Furthermore, several studies have suggested that the shorter average path length of a bacterial community network can facilitate efficient and rapid communication among members within a community, so the whole community could make quick responses to environmental changes [67]. As such, the rhizosphere bacterial community in CW may respond rapidly to environmental fluctuation resulting from daily pollutants input, enabling promptly positive feedback between plants and their associated microbes. Within microbial community network, a module can be defined as a functional group that shares the same ecological niche and play important role in element cycling [68]. Thus, the modularity of the bacterial community network may reflect habitat heterogeneity, resource partitioning, and functional association [69]. Although the lower modularity was observed in the rhizosphere bacterial community network of CW compared to that of NW (Table 2), such responses could potentially be common given that high-nutrient conditions in CW may be less prone to promote bacterial niche differentiation [70]. Additionally, a previous study revealed that the interactions among taxa were increased along with the aggravation of pollution, and this process promoted the connectivity among modules but decreased the modularity of the network [71]. And it has been reported that the higher-level functions can be achieved by connecting modules together [72]. Therefore, the *P. australis* bacterial community network of CW strengthens the connections among diverse functional modules in the rhizosphere, which might help to maximize the effects of *P. australis* on water purification.

Hub taxa, identified in each bacterial community network including module hubs and connectors, were often regarded as keystone taxa due to their essential roles in maintaining the network structure and ecological functions [23]. In this study, our results showed that rhizosphere/bulk-enriched bacterial taxa could act as keystone species driving the assembly of the corresponding bacterial community co-occurrence network. We found 11 keystone OTUs of the bulk bacterial community network in NW were identified as bulk-enriched OTUs (Table S3). Two of these, the genera *Gemmatimonas* and *Terrisporobacter*, have been reported as important contributors for nutrients transformation [73, 74]. Three keystone OTUs of the rhizosphere bacterial community network in CW were identified as rhizosphere-enriched OTUs. The genus *Rhodoferax* has the potential capability for pollutants degradation and maintains cooperative metabolic associations with other species [75]. These findings suggested that enriched bacterial taxa found in wetlands could play a critical ecological role not only in the nutrient cycling and pollutants degradation but also in the maintenance of the network structure through frequently co-occurring with other bacterial taxa.

Conclusion

This study provides a detailed characterization of diversity, composition, and co-occurrence patterns of rhizosphere and bulk bacterial communities of *P. australis* between NW and CW sediments. Consistent with our hypothesis, the results indicate that compositional differences in the sediment bacteria were largely driven by wetland types; and the bacterial community of rhizosphere selectively recruitment in CW was different from that of NW. Additionally, our work unveiled plant selectively recruitment for specific taxa and the divergence in the complexity of rhizosphere bacterial community networks between CW and NW. Those rhizosphere-enriched bacterial groups can impact elements cycling and potentially affect water purification, as well as maintain interactions within the bacterial community in wetlands. Future researches could use these candidate bacterial consortia for the synthetic community to further test the functional roles of these bacterial taxa. Our findings have important implications for understanding the status and roles of the rhizosphere bacterial community in maintaining the ecological functions of CWs.

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Declarations

Conflict of Interest The authors declare no conflict of interest.

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