

Mangrove plants improve predominant microbiota in constructed wetlands for wastewater treatment

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Abstract

Background

Two wetlands planted with *Kandelia candel* and *Aegiceras corniculatum* and one wetland without plantation were constructed for stable wastewater treatment since 2005. The impact of mangrove plants on the microbiota of wetlands was studied due to their higher efficiency of wastewater treatment.

Results

Microbiota of each wetland were explored through high-throughput sequencing and their relationships were predicted based on Spearman metrics. Planted wetlands had higher microbial diversities and more similar microbial communities. Five phyla were significantly enriched in planted wetlands, including Acidobacteria, Nitrospirae, Actinobacteria, Gemmatimonadetes and Elusimicrobia. Twelve phyla of other microbes accounting for 1.85% of the total relative abundance showed significantly higher abundance in the unplanted wetland. Planted wetlands shared more similar microbial community structure distinguished from that of the unplanted one. Additionally, distinct microbial interactive pattern could be observed in planted wetlands.

Conclusions

Based on their microbial compositions and interactive patterns, it will be reasonable to illustrate the higher wastewater purification ability of planted wetlands. Research here also supplies useful information for the potential of applicable combination of certain kinds of microbes for wastewater treatment.

Background

Mangrove wetlands are intertidal ecosystems located in subtropical and tropical coastal regions between terrestrial and marine environments (Feller et al., 2010). They harbored great biodiversity and were characterized as one of the most productive ecosystems (Cabral et al., 2016; de Sousa et al., 2017; Yun et al., 2017). Carbon, sulfur, nitrogen, phosphorous cycles are rather active in mangrove forests (Bai et al., 2013; Pires et al., 2012; Zhang et al., 2015). However, mangrove wetlands usually suffered from deforestation and diverse kinds of contamination due to their special geographic location and rapid process of urbanization and industrialization (Zanaroli et al., 2015; Peixoto et al., 2011; Sandilyan and Kathiresan, 2014; Wang et al., 2013; Zhang et al., 2014). Previous research had proved that mangrove wetlands were able to accumulate metal and organic pollutions effectively (Zhang et al., 2014; Zhao et al., 2012). In addition to the adsorption of pollutions by mangrove plants, microbial communities were regarded as the predominant players in the clean-up process of nutrient pollutants (Tam and Wong, 2008; Yu et al., 2005).

Due to the fact that research conducted in natural environments lost stable control and data restricted to pot experiments could not reflect the population effect of mangrove plants. Two mangrove wetlands planted with *K.candel* and *A.corniculatum* separately together with one wetland without any plant were constructed for treatment of municipal wastewater since 2005(Yang et al., 2008). Wastewater influent with relative high content of nutrient was under control and all wetlands were reported to efficient and stable in sewage treatment for more than 10 years especially for planted ones(Tian et al., 2017). Similar studies on removal of organic and inorganic pollutants in different constructed wetlands planted with various kinds of plants had gained great achievement(Verlicchi and Zambello, 2014; Zhang et al., 2015). Planted wetlands were more efficient in wastewater treatment than unplanted ones which also related with plant species(Keffala and Ghrabi, 2005; Leung et al., 2016). In addition, constructed wetlands were also reported to effectively clean human pathogens and faecal indicators which would be of great threaten for human health(Nasser, 2016; Wu et al., 2016). However, reports also revealed that sediments were mainly responsible for pollutants accumulation but not plants(Arroyo et al., 2013). Microbes in sediments were the leading players in cleaning pollutants(Tam and Wong, 2008).

In fact, microbes in mangrove wetlands are not only diverse in community structure, but they are also of various functions as single isolate or in microbial consortium. Combined culture of bacterial strains isolated from mangrove wetlands were able to degrade recalcitrant and toxic pyrene at higher rate than that when individual isolates were applied(Wanapaisan et al., 2018). Dehalogenating bacteria were enriched by spent mushroom substrate derived biochar applied in anaerobic mangrove sediment which was supposed to be responsible for higher debromination efficiency of 2,2',4,4'-tetrabromodiphenyl ether(Chen et al., 2018).

Structure of microbial communities would be shaped in different environments and distinct microbes were reported to be enriched in special conditions((Dos et al., 2011; Gomes et al., 2011; Hazen et al., 2010). Plant species also exerted influence on the microbial composition(Berg and Smalla, 2009; Yun et al., 2017). Such transitional microbial communities could also be characterized as sensitive indicators of the ecosystem(Tian et al., 2008). Certain microbes with increased abundance in distinct environments were speculated to be of application potential for bioaugmentation strategy in bioremediation (Dos et al., 2011; Wang et al., 2016). Due to the higher efficiency of mangrove planted wetlands in nutrient removal comparing with the unplanted one, microbiota in these three wetlands were systematically explored by high-throughput sequencing. Diversity, composition and relative abundance of microbiota will be analyzed to explain whether they were responsible for the elimination of nutrients in these wetlands. Whether special microbiota had been enriched or decreased among planted and unplanted wetlands will be also discussed in this research.

Methods

Wetlands and soil sampling

Two mangrove wetlands planted with *K. candel* or *A. corniculatum* and one unplanted wetland were constructed individually in Futian Natural Nature Reserve, Shenzhen Bay, Shenzhen, China (22°32'N, 114°05'E). These three wetland belts were separated at a distance of 0.5m between each two neighboring ones. Each belt was designed to the same size of 33m (length) ×3m (width) ×0.5m (depth) and divided into five parts, including one inlet zone (1m length), one outlet zone (1m length), two planted or unplanted zones (15m length) and one transition zone (1m length) for connecting them together as described before (Yang *et al.*, 2008; Tian *et al.*, 2017). The planted zones in each belt were filled with stone, gravel and sand sequentially from the bottom to the top surface with an depth of 20cm, 20cm and 10cm, respectively. So was for the unplanted belt. The other three parts in all the belts were empty without filling anything. The wetlands were applied for municipal wastewater treatment since August, 2005. After sedimentation for 1h, municipal wastewater was discharged into the inlet parts of the planted or unplanted wetlands. The hydraulic loading volume for each belt was 5m³d⁻¹ and the retention time for wastewater in wetlands was 3d. These three wetlands were able to purify the municipal wastewater stably since then, with the planted ones owing significantly higher capacity for wastewater treatment. The *A. corniculatum* and *K. candel* wetlands had 34.7% and 16.9% higher capacity of maximum total nitrogen (TN) removal than the unplanted one (Tian *et al.*, 2017).

Surface sand samples at a depth of 5-10cm at each belt were collected in May, 2017. Surface sand at a depth of 0-5cm was discarded since they were not submerged under the water surface for water treatment. For comprehensively and randomly investigation of microbiota involved in wastewater treatment, samples were collected at the rims of inlet, transition and outlet zones which were marked as FS, MS and LS accordingly. Every six replicates of sand samples were collected at FS, MS and LS sites in each wetland belt. Therefore, a total of 54 sand samples were collected and stored at -80°C for further experiment.

DNA extraction

Aliquot 0.5g of soil samples were prepared to extract genomic DNA using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA) according to the manufacturer's instruction. Three parallel copies of DNA were obtained in sterile water and mixed into one single tube. The DNA quality and concentrations were confirmed by 1.2% agarose gel electrophoresis and NanoDrop 2000 microvolume Spectrophotometer (Thermo Fisher Scientific, USA). The qualified DNA was stored at -80°C for PCR amplification.

16S-V4 PCR amplification and Illumina sequencing

The V4 region of bacterial 16S rRNA was amplified with prokaryotic primers 515F(5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (Antoniou *et al.*, 2015). Primers were labeled with a unique 12 nt barcodes. The reaction mixture contained 25ng of template DNA, 25µL Premix *Taq* DNA polymerase mixture (TaKaRa, Japan), 5µL of each primer (5µM) and 10µL sterile water. The PCR procedure was performed on ABI GeneAmp® 9700 thermal cycler (Thermo Fisher Scientific, USA). The amplification procedure was initially denaturated at 95°C for 3min, followed by 28

cycles of 95°C for 30s, 55°C for 30s, 72°C for 45s, with a final extension at 72°C for 10min. The amplified products were pooled and analyzed on 1.2% agarose gel electrophoresis to confirm the purity of the PCR products. The bands with right size were excised and purified using E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, USA). Equal amounts of purified products were combined and sent for pair-end sequencing on Illumina Miseq 2000 platform (Illumina Inc., USA) at Major Bio-Pharm Technology Co.Ltd, Shanghai.

Sequence analysis

In order to obtain qualified sequence, raw sequence data were processed using Quantitative Insights Into Microbial Ecology pipeline version 1.8.0 (Caporaso *et al.*, 2010). All sequence reads were strictly assigned based on their barcodes. Only sequences with high quality (average base quality score ≥ 30 , sequence length ≥ 200 bp and without any ambiguous base) were retained for downstream analysis. High quality sequences were clustered into operational taxonomic units (OTUs) at the 97% sequence identity level after removing the barcodes. OTU representative sequences which were the most abundant sequence from each OTU were then assigned with taxonomy using Ribosomal Database Project classifier (Wang *et al.*, 2007). A total of 338,6291 sequence reads remained with an average sequence length of 273bp. All the sequences were submitted to National Center for Biotechnology Information Sequence Read Archive with an accession number SRP149551. All samples were normalized to an even sequence depth of 40,909 reads. Shannon indices and rarefaction curves of each sample were analyzed using QIIME to evaluate the complexity of bacterial communities within each sample. Principal coordinate analysis (PCoA) based on weighted unifracs distances was calculated for classification of bacterial communities among different wetlands.

Statistical analysis

Statistical analysis was performed by using SPSS software (Version 16.0, SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) followed by Tukey post-hoc test at a significant level of $p \leq 0.05$ was carried out to determine whether there were significant differences of microbial abundance among different wetlands. In order to predict the relationship of microbial communities in each different wetland, co-occurrence networks of microbiota in them were constructed individually based on the Spearman correlation coefficients. Microbes at phylum level in all of the samples were involved in the analysis. Only microbes with strong relationships ($R \geq 0.5$ or $R \leq -0.5$, $p \leq 0.05$) could be shown in the co-occurrence networks visualized by Cytoscape 3.5.1 (Shannon *et al.*, 2003).

Results

Diversity of microbiota in different constructed wetlands

A total of 3386291 high-quality sequences were generated by Illumina sequencing the V4 region of the 16S ribosomal RNA gene of bacteria from 54 soil samples collected from constructed mangrove or unplanted wetlands. After barcodes removal, quality filtering and normalization, 2209086 sequences

remained and were grouped into OTUs at 97% similarity level. These OTUs were taxonomically grouped into 56 phyla, 11 of them exceeded 1% of relative abundance. Both rarefaction and Shannon diversity indices curves reached stable values, which suggested that most of the microbial diversity had been successfully captured (Fig 1, Supplementary Fig S1). Based on rarefaction curves, microbiota in *K. candel* (5616) wetland had the highest number of microbial OTUs followed by that in *A. corniculatum* (5493) and unplanted (5363) wetlands, respectively. Among which, about 78.3% (4718 of 6026) of the OTUs existed in all the three different soil samples and about 16.7% (1008 of 6026) of them co-existed in two of the three samples (Supplementary Fig S2). Planted wetlands shared more similar microbial communities than with the unplanted wetland due to that 91.3% of the microbial OTUs of the two planted wetlands was identical (Table 1). And 5.0% (299 of 6026) of the total OUTs solely appeared in one of the three wetlands, of which microbiota in unplanted wetland had the absolutely highest number of unique OTUs (219) than that both in *K.candel* (12) and *A.corniculatum* (68) wetlands.

Composition of microbiota in different constructed wetlands

Proteobacteria, Acidobacteria, Chloroflexi, Nitrospirae, Planctomycetes, Verrucomicrobia, Bacteroidetes, Actinobacteria and Ignavibacteriae were the top ten most abundant bacterial phyla in all of the soil samples, except for unclassified microbes which accounted for 13.1% of the total relative abundance. These nine identified microbiota at phylum level accounted for 78.2% of the total relative abundance. While in different wetland soil samples, the top 10 most abundant microbes were not always the same as that of the whole one. Gemmatimonadetes and Firmicutes were included in the top 10 most abundant microbes in *A.corniculatum* soil samples other than Bacteroidetes and Ignavibacteriae, while Gemmatimonadetes was one of the top 10 most abundant microbes in *K.candel* soil samples but not Ignavibacteriae (Table 2). Of the top 10 most abundant microbes in *A.corniculatum* and *K.candel* soil samples, Acidobacteria, Nitrospirae, Actinobacteria and Gemmatimonadetes had significantly higher relative abundance than that in unplanted soil samples. In addition, Elusimicrobia was another phylum of microbe with significantly higher relative abundance both in *A.corniculatum* and *K.candel* soil samples than that in unplanted ones, although it only accounted for less than 0.03% of the total microbial relative abundance. Bacteroidetes was the only one of the top 10 abundant phylum of microbes with significantly higher relative abundance in unplanted wetland than the two planted ones. Additionally, other 12 phyla of microbes were also of significantly higher abundance in unplanted wetland compared to the other two planted wetlands (Supplementary Table S1). However, they only accounted for about 1.85% of the total relative abundance. Interestingly, some microbes liable to be significantly enriched in certain kind of planted wetland were also been discovered here. Verrumicrobia, one of the top 10 most abundant microbes, was of significantly higher relative abundance in *K.candel wetland* than that in the other two wetlands nomatter whether they were planted or not. Three phyla of microbes with low relative abundance (less than 0.1% of the total relative abundance), Chlamydiae, TM6-Dependentiae and Candidatus-Berkelbacteria, were specially significantly enriched in *A.corniculatum* wetland.

Classification analysis of microbiota in different constructed wetlands

Although some soil samples belonging to mangrove wetlands were clustered together with those from unplanted wetland, most of them (28 of 36) were uniquely clustered into one separate group based on weighted-unifrac distance (Fig 2). Above results confirmed that unplanted and planted wetlands differed greatly in bacterial community structure. The planted wetlands shared relatively similar bacterial community structure.

Co-occurrence network of microbiota

Spearman correlation networks were constructed based on coefficient to identify the potential co-occurrence and interaction patterns, which were connected among the 5 phyla of microbes with significantly higher abundance both in *A.corniculatum* and *K.candel* samples with those microbes (13 phyla) only significantly enriched in unplanted wetland (Fig 3). As to those microbes with significantly higher abundance in both planted wetlands, they built positive connections among each other if there were, no matter they existed in planted or unplanted wetlands. Only negative correlations were observed among them with those microbes with significantly higher abundance in unplanted samples in *A.corniculatum* wetland. This was not the same microbial interactive pattern appearing in *K.candel* samples. In both the planted wetlands, microbes with significantly higher abundance in unplanted wetland just positively correlated with each other. As to Bacteroidetes, one of the top 10 phylum of bacteria with significantly higher relative abundance in unplanted wetland, only negatively correlated relationship could be build with the five phyla of microbes of significantly higher abundance in planted wetlands if there were. And Bacteroidetes positively correlated with Fibrobacteres and Spirochaetae irrespective of plantation or not in all the wetlands. While in the microbial correlated network of unplanted wetland, no determined co-occurrence or interactive mode could be checked among all the microbes.

Discussion

Diversity, composition and correlated networks of microbiota in constructed *K.candel*, *A.corniculatum* and unplanted wetlands for municipal wastewater treatment since 2005 were explored by high-throughput sequencing. Microbiota of the planted wetlands were both of higher diversity than that of the unplanted one. Five phyla of microbes, including four of the top 10 most abundant microbes in all the wetlands, were both significantly enriched in the planted wetlands. Additional typing analysis showed that microbiota of planted wetlands were clustered together and separated away from that of the unplanted one. Above results indicated that microbiota in planted wetlands were much different from that of the unplanted one and recruitment effect of *K.candel* and *A.corniculatum* on relative high abundant microbiota was rather significant. Such effect of mangrove plants on microbial diversity had also been reported previously. Chen et al. reported that microbiota in sediment increased remarkably after mangrove succession by analysis of phospholipid fatty acid (PLFA)(Chen et al., 2016). Plantation of bare wetlands with mangrove plants would cause changes in habitat due to the additional nutrient availability from litter decomposition and plant secretions, leading to the shift of microbial community(Kristensen et al., 2008; Navarrete et al., 2015; Sahoo and Dhal, 2009). Previous studies also pointed out that different microbial communities corresponded to different mangrove species(Dias et al., 2010; Sahoo and Dhal, 2009). In

this research, some distinct bacteria only appeared with significantly higher relative abundance in one of the two different mangrove wetlands, such as Chlamydiae, TM6_Dependentiae, Candidatus_Berkelbacteria in *A.corniculatum* wetland and Verrumicrobiota in *K.candel* wetland. Above results supported the conclusion that planted wetlands shared the more similar bacterial communities than that of unplanted one. Mangrove plants here were partially reasonable regarded as host with selectivity for microbial communities in such wetlands due to their contribution of extra nutrients. Indeed, the selectivity of microbial communities seems to be a commonly natural phenomenon among various host species and even exists in different compartments of one plant(Astudillo-Garcia et al., 2017; Bjork et al., 2013; Edwards et al., 2015; Erwin et al., 2012).

These two constructed mangrove wetlands had been proved to be able to stably treat municipal wastewater and remove TN since 2005(Tian et al., 2017; Yang et al., 2008). Some native predominant microbes simultaneously with increasing relative abundance and N removal metabolism potential were discovered here, including Proteobacteria, Acidobacteria, Nitrospirae and Actinobacteria. They were reported to involve in denitrification or nitrate and nitrite reduction which would both reduce N component in natural or artificial environments(Barta et al., 2017; Lin et al., 2014; Vishnivetskaya et al., 2013; Wang et al., 2017; Ward et al., 2009; Xing et al., 2017). Three of them was of significantly higher relative abundance in both of the planted wetlands than the unplanted one, leading to the reliable explanation of the high efficiency of TN removal in all of the three wetlands, especially in the two mangrove planted wetlands.

Fibrobacteres and Spirochaetes were two phyla of bacteria with cellulose degradation ability(Leschine, 1995; Ransom-Jones et al., 2012). They were supposed to be of higher relative abundance in both of the two planted wetlands, due to that mangrove plants would supply fallen leaves with greater level of cellulose compared to the unplanted one. Conversely, they were of higher relative abundance in the unplanted wetland comparing with the two planted ones, especially for Spirochaetes (Table 3). By analysis of the microbial correlated network (Fig. 3), both Fibrobacteres and Spirochaetes correlated negatively with those microbes of significantly higher abundance in planted wetlands in direct or indirect manner in all of the three wetlands. Microbial correlated networks based on spearman or pearson metrics were proved to be able to reveal the possible communication among microbial communities and valuable for identifying unexplored microbial associations(Edwards et al., 2015). They were also applicable in confirmation of theoretical relationships among microbes and environmental or physiological factors, which is of great value for clinical diagnosis and evaluation of variances related with microbial community shift in natural environment(Bajaj et al., 2015; Wedin et al., 2016). However, by building microbial correlated networks, Cardinale *et al.* suggested that microbes important for structuring the microbial association could be determined regardless of their relative abundance and phylogenetically close species would function differently in diverse ecological environments(Cardinale et al., 2015). This is in accordance with results in this research (Table 4). By building such microbial correlated networks, it could at least partially reflect the hidden competitive or synergistic interactions among microbes of different abundance in various environments. Thus it was reliable to illustrated that although there was higher level of cellulose supply in planted wetlands, Firbobacteres and Spirochaetes could not be

enriched or even of less relative abundance than that in unplanted wetland. The negative correlations among them and microbes of higher relative abundance in planted wetlands indicated the greater suppression effect of the later ones on Fibrobacteres and Spirochaetes communities development. The distinct microbial relationships in planted wetlands different from the unplanted one showed that plantation of mangrove species would not only change the composition of microbes in wetlands, but the interactive modes among them also differentiated which could be considered as a sign of function development.

Conclusions

The total diversity and composition of microbiota in two mangrove plant wetlands and one unplanted wetland were unaltered here. The plantation of bare wetlands with *K.candel* and *A.corniculatum* reshaped the microbiota pattern and lead to native predominant denitrifiers with increasing relative abundance, including Proteobacteria, Acidobacteria, Nitrospirae and Actinobacteria, potentially accounting for TN removal. Microbial communities with cellulose degradation abilities were less abundant in planted wetlands than that in the unplanted one due to the competitive effect of highly abundant microbes in the planted wetlands. Research here could be considered as a reference for applicable combination of certain kinds of microbes for wastewater treatment.

Declarations

Permission for sample

These wetlands were constructed by Tam, one of the authors, so we don't need to get any other permission.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All the raw sequence data has been submitted to NCBI with an accession number SUB4105853.(
<https://submit.ncbi.nlm.nih.gov/subs/sra/>)

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table1 Ratios of common OTUs among planted and unplanted wetlands

Group	KC-AC	KC-B	AC-B
Ratio	91.3%	84.3%	80.5%

AC: *A. corniculatum*; B: Blank; KC: *K. candel*

Table 2 Top 10 most abundant microbes at phylum level in AC, B and KC samples

Number	AC	B	KC
1	Proteobacteria	Proteobacteria	Proteobacteria
2	Acidobacteria	Unclassified_k_norank	Unclassified_k_norank
3	Unclassified_k_norank	Chloroflexi	Acidobacteria
4	Chloroflexi	Acidobacteria	Chloroflexi
5	Nitrospirae	Planctomycetes	Nitrospirae
6	Planctomycetes	Bacteroidetes	Verrucomicrobia
7	Actinobacteria	Nitrospirae	Planctomycetes
8	Verrucomicrobia	Verrucomicrobia	Actinobacteria
9	Gemmatimonadetes	Ignavibacteriae	Bacteroidetes
10	Firmicutes	Actinobacteria	Gemmatimonadetes

Species marked as bold font are those with significantly higher relative abundance in planted wetlands than that in the unplanted one.

Table 3 Relative abundance of Fibrobacteres and Spirochaetae in planted and unplanted wetlands

Group Species	AC	KC	B
Fibrobacteres	252	383	849
Spirochaetae	4711	4187	8808

Table 4 Direct or indirect correlations among microbes of significantly higher relative abundance in planted wetlands and Bacteroidetes with Fibrobacteres and Spirochaetes in all kinds of wetlands

Species	AC		KC		B	
	Fibro	Spiro	Fibro	Spiro	Fibro	Spiro
Acidobacteria	-	+	-	-	-	+
Nitrospirae	-	-	-	-	-	-
Actinobacteria			-	-	-	-
Gemmatimonadetes	+	+	-	-	-	-
Elusimicrobia	-	-	-	-	+	-
Bacteroidetes	+	+	+	+	+	+

Fibro: Fibrobacteres; Spiro: Spirochaetae

+: direct correlations; -: indirect correlations; blank: no significant correlation

Figures

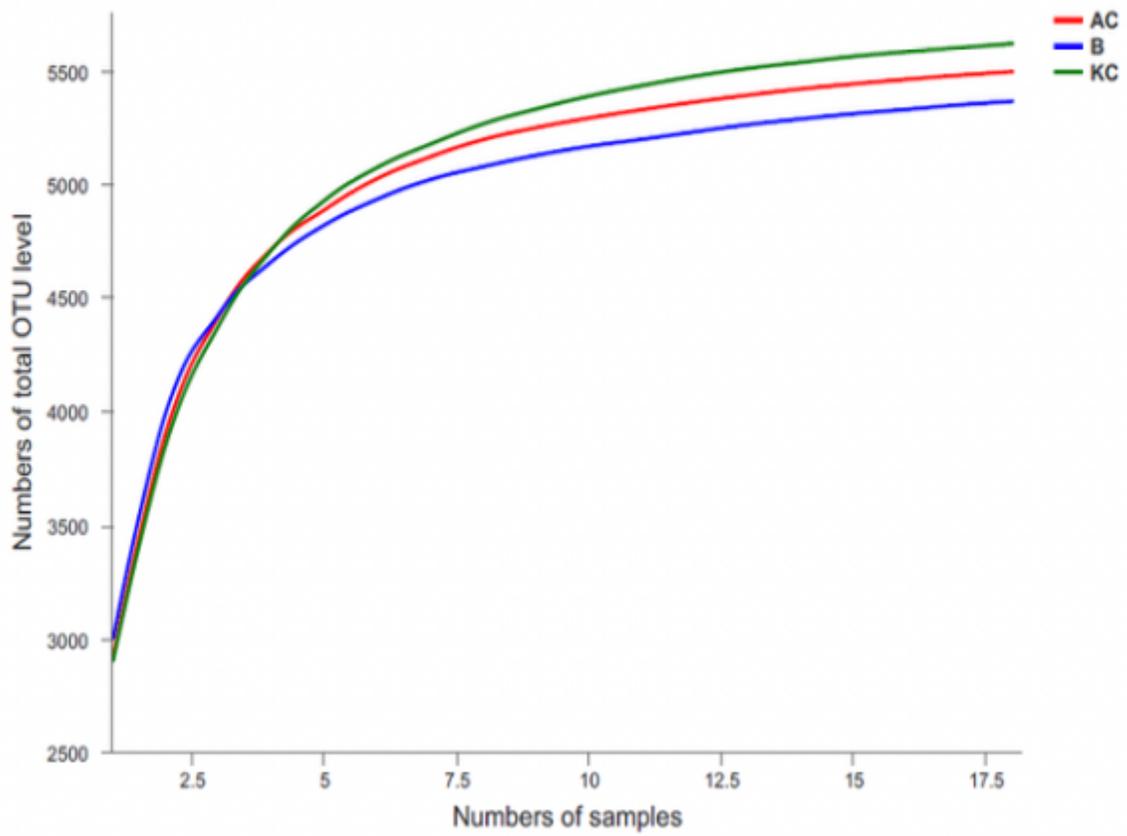


Figure 1

Pan analysis of the total microbiota in different samples. AC: *A. corniculatum*; B: Blank; KC: *K. candel*

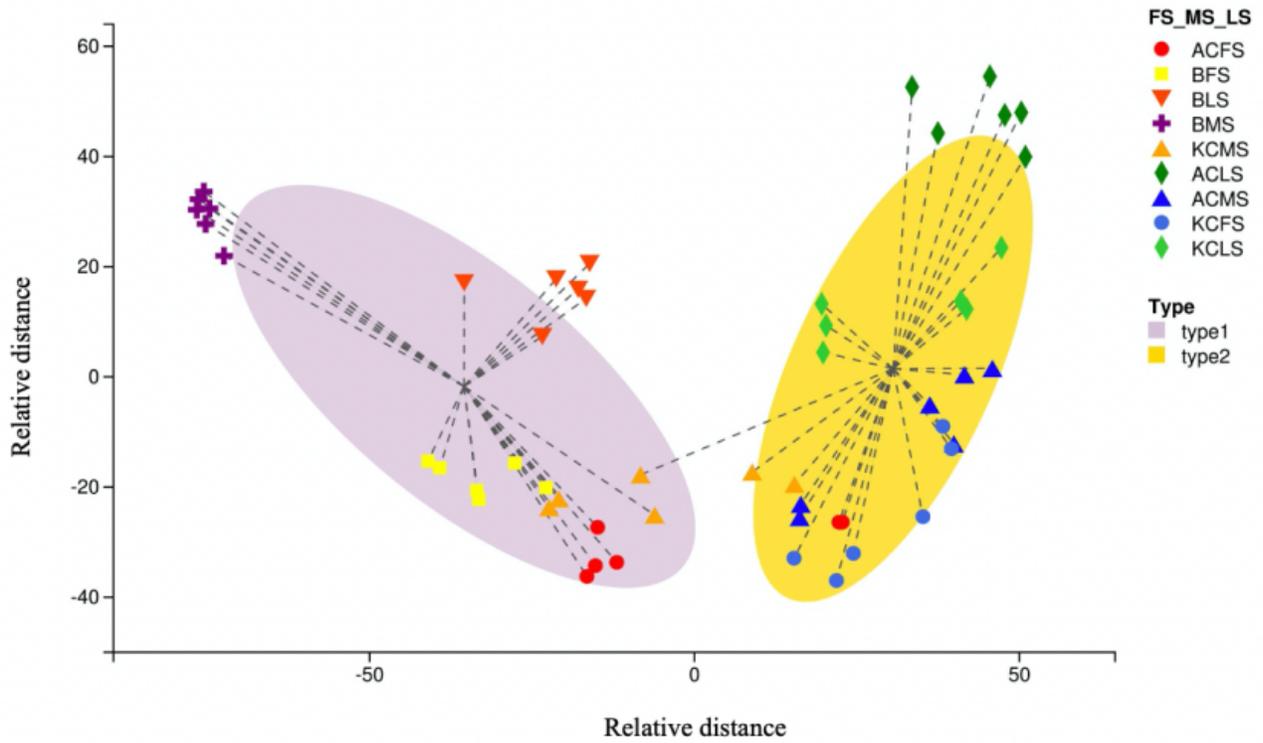


Figure 2

Principal coordinate analysis for classification of microbiota in different samples. Ellipses with distinct color represent different types of microbial communities.

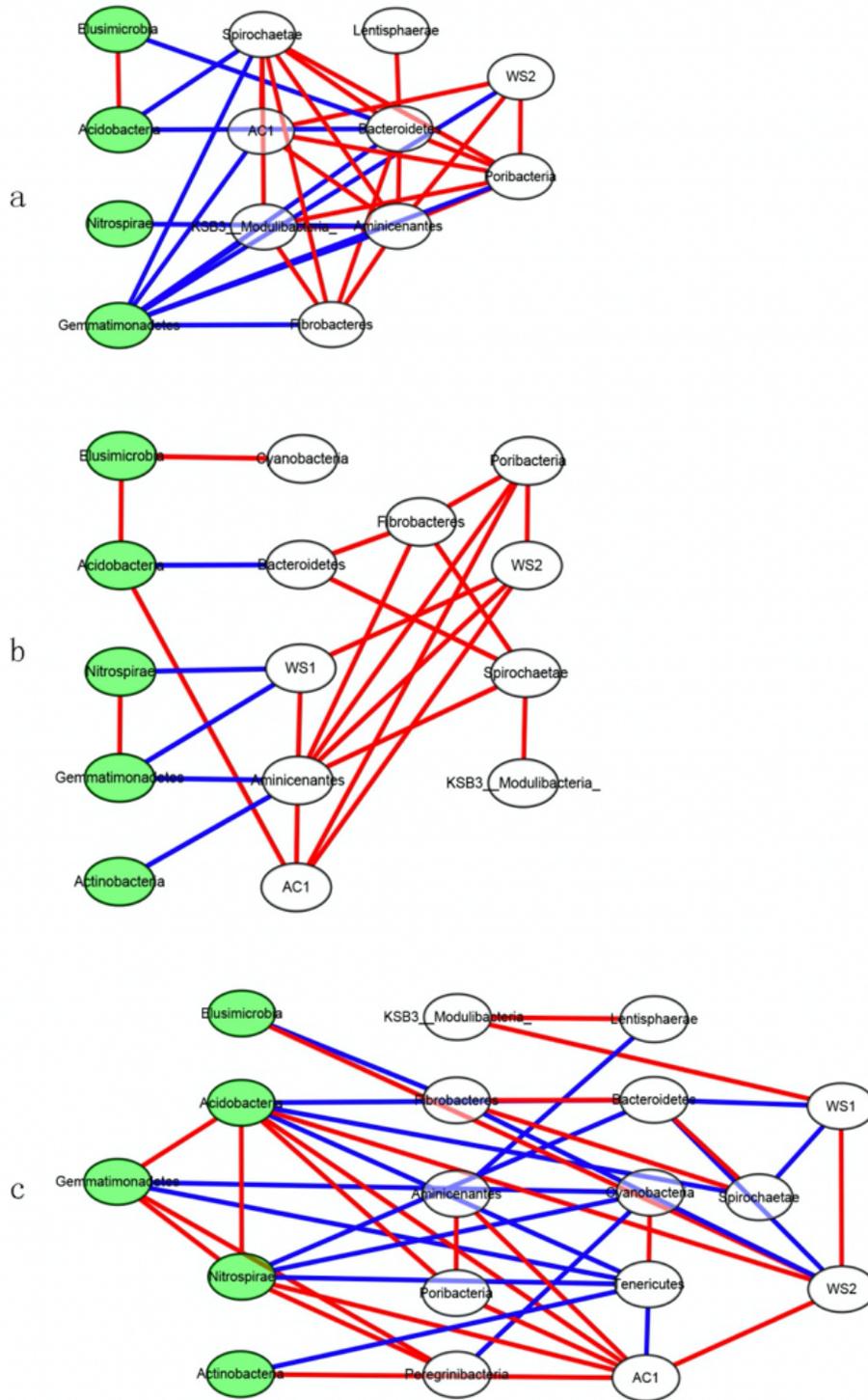


Figure 3

Co-occurrence networks of certain microbiota in different wetland samples. a, b, c: Co-occurrence networks of microbiota in *A. corniculatum*, *K. candel* and unplanted wetlands, respectively. Elliptical nodes represent microbes at phylum level; the red connecting lines indicate positive relationships among microbes, while the blue connecting lines indicate negative ones; green nodes represents

microbes with significantly higher relative abundance in both planted wetlands, while white nodes represent microbes with significantly higher relative abundance in unplanted one.

Supplementary Files

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