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Nitrogen Retention in Mesocosm Sediments Received Rural Wastewater Associated with Microbial Community Response to Plant Species

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Received: 15 September 2020; Accepted: 27 October 2020; Published: 29 October 2020

Abstract: Vegetated drainage ditches (eco-ditches) have drawn much attention in recent years for the ability to remediate diffuse contaminants in rural wastewater through sediment retention, plant uptake and interception, and microbial metabolic activities. However, the effect of plant species on microbial community structure and nitrogen (N) retention in ditch sediment remains poorly understood. In this study, mesocosm plastic drums were planted with eight plant species commonly found in ditches and nurtured with wastewater for 150 days. Sediment total nitrogen (TN) was greatly increased after 150-day nurturing with rural wastewater, from 296.03 mg·kg^{-1} (*Iris japonica Thunb*) to 607.88 mg·kg^{-1} (*Acorus gramineus O*). This study also presents the effect of different plant species on sediment microbial communities, thus providing insight into N removal mechanisms in eco-ditch. Fifty-eight differentially abundant taxa were identified, and sediment microbial community structure for no plant (CK), Acg, *Canna indica* (Cai), and *Typha latifolia* L. (Tyl) was primarily linked to sediment NH_4^+-N and TN. Extremely small proportions of ammonia oxidizing bacteria (AOB) and nitrifying bacteria were detected for all treatments, but large proportions of Crenarchaeota, which comprises the widely existent ammonium oxidized archaea (AOA), were found in CK, Acg and Cai. The abundance of Nitrosotalea from Crenarchaeota presented positive correlations with sediment NH_4^+-N contents and ammonia oxidation function predicted by Faprotax, indicating Nitrosotalea might be the dominant ammonium-oxidizing microbes in sediment samples. The probable NH_4^+-N removal pathway in wastewater sediment was through a combined effect of AOA, nitrifying bacteria, and anammox.

Keywords: microbial community; rural domestic wastewater; eco-ditches

1. Introduction

Rural wastewater derived from agricultural and domestic activities is a major source of water pollution. The pollutants from rural wastewater include high concentration of organic matter and a certain amount of N, which may cause eutrophication in natural water bodies when the wastewater is discharged directly without treatment [1]. Conventional wastewater treatment processes are prohibitively expensive and not entirely feasible for widespread application in rural areas [2,3]. Thus, ecological technologies have gained increasing attention in the past two decades for the successful and

cost-effective treatment of rural wastewater compared with conventional treatment methods [3–5]. Eco-ditches, transformed from conventional agricultural drainage ditches with drainage channels, substrate, vegetation, and flow control facilities, are beneficial management practices (BMPs) under close examination for nutrient mitigation [6–9]. More recently, the nitrogen (N) mitigation mechanisms of eco-ditches has been investigated. As with similar mechanism of surface-flow constructed wetland, eco-ditches could mitigate pollutants before entering into downstream water [10]. The main mechanisms for N removal in eco-ditches are plant uptake and interception, sediment retention, and microbial metabolic activities [6,11].

Plants are generally considered to mitigate N pollutant by direct uptake from water, increasing hydraulic retention time of wastewater, providing oxygenation micro-zone by radial loss of oxygen through the roots, and providing surface area for microbial biofilms [12,13]. Previous studies have suggested plant uptake accounts for 45–86% of total N removal [7–9]. Plants are also the primary drivers for microbial community composition variation in sediment from ecological treatment [14]. Each plant species is supposed to select specific microbial populations because of the variation in the composition of root exudates from each species, and affects the relative abundance of microorganisms in rhizophere [15]. Briones et al. [16] identified cultivar-specific differences for AOB in rice rhizospheres. Bremer et al. [17] reported that different species of non-leguminous grassland plant affected nirK-type denitrifier community composition directly through root exudates. Although the microbial community plays a fundamental role in regulating nutrients transformation and degradation [18], the effect of plant species on microbial composition and metabolic activities in ditch sediment remains poorly understood. Moreover, the microbial community is very sensitive to environmental variations, and the community composition varies with the alterations of environmental factors, including pH, temperature, dissolved oxygen (DO), N substrate, labile carbon, etc. [19,20]. Therefore, the microbial community composition might be a good indicator of the status of eco-ditch system. Here, we combined mesocosm plastic drum sediment (MPDS) experiment and MiSeq 16S rRNA gene sequencing to explore the underlying microbial mechanism of nitrogen removal from rural wastewater. We aimed to investigate (i) the effect of plant species on microbial diversity in eco-ditch sediment received rural wastewater and (ii) the link between microbial clades and N removal.

2. Materials and Methods

2.1. Plant Species

Eight common aquatic and terrestrial plant species in the middle and upper reaches of Yangtze valley were selected, including *Phyllostachys heteroclada F.* (Phh), *Acorus gramineus O.* (Acg), *Canna indica* (Cai), *Myriophyllum verticillatum* (Myv), *Iris japonica Thunb(Ijt)*, *Eichhornia crassipes* (Eic), *Rumex patientia* (Rum), and *Typha latifolia* L. (Tyl). Plant species were collected locally from natural ditches in February 2016.

2.2. Mesocosms Plastic Drum Sediment (MPDS) Experiment

The MPDS was designed to evaluate the effect of different plant species on pollutants removal from rural wastewater. The drums were 55 cm (h) × 28.5 cm (d) with a vertical perforated PVC pipe installed in the centre of each plastic drum to pump wastewater [9]. The cultivation experiment of MPDS was conducted in a greenhouse constructed of polyethylene film at Yanting Agro-Ecological Station of Purple Soil, Chinese Academy of Sciences. The selected plant species were nurtured with pond water for approximate 2 weeks until the plants developed root systems. Healthy and uniformly sized young plants with an average height of 10–15 cm were then rinsed and transplanted into the MPDS drums at a density of 10–12 plant/m^2. The drums were filled with 30-cm depth of sediment collected from natural ditch around the station. Rural wastewater obtained from the ditches was pumped through pipes into each drum. The wastewater was added once per week during the experiment to compensate for evaporation and maintain a constant water level (35 cm). The properties of pond water,

rural wastewater, and sediment are shown in Table 1. Each plant treatment has three replicates, and two control (CK) drums were left unplanted. Air temperatures in the plastic house were maintained between 14–38 °C during the experimental period. The plants flourished and the sediment microbes settled after being acclimated for 150 days.

Table 1. Characteristics of pond water, rural wastewater and sediment.

Parameter	Pond Water	Rural Wastewater	Sediment ($mg \cdot kg^{-1}$)
pH	7.85	8.01	-
DO	7.6	0.35	-
TDS	266	885	-
EC	384	1452	-
TN ($mg \cdot L^{-1}$)	8.86	58.64 ± 2.21–65.42 ± 3.52	575.65 ± 25
TP ($mg \cdot L^{-1}$)	0.58	4.87 ± 0.25–5.92 ± 0.58	498.47 ± 25
NH_4-N ($mg \cdot L^{-1}$)	0.38	22.73 ± 1.75–28.42 ± 2.07	56.24 ± 1.25
NO_3-N ($mg \cdot L^{-1}$)	0.92	1.97 ± 0.20–2.84 ± 0.29	15.24 ± 1.42

TDS: total dissolved solids; EC: electrical conductivity; TP: total phosphorus; NH_4^+-N: ammonium nitrogen; NO_3^-N: nitrate nitrogen.

2.3. Sediment Sampling and Chemical Analyses

Sediment samples were collected at the end of the cultivation experiment from each MPDS system, and vegetable matter was discarded. One subsample was stored at 4 °C for chemical analyses, while another subsample was stored at –80 °C for molecular analysis. NO_3^-N and NH_4^+-N were extracted with 2 M KCl and analyzed using a continuous-flow analyzer (Model AA3; Bran + Luebbe, Norderstedt, Germany). TN was determined by semi-micro Kjeldahl digestion using Se, $CuSO_4$, and K_2SO_4 as catalysts. Dissolved organic carbon (DOC) was extracted with 0.5 M K_2SO_4 and analysed using a TOC-5000 analyzer (Shimadzu, Kyoto, Japan). Organic Carbon was determined based on dichromate oxidation and titration with $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. The pH was measured from fresh soil-water suspensions (1:2.5 *w/v*). The sediment moisture content was calculated by determining the weight loss of sediment sample after drying at 105 °C for 24 h. TP was analyzed using the alkaline potassium persulfate digestion-ultraviolet spectrophotometric method.

2.4. DNA Extraction and MiSeq Sequencing of 16S rRNA Gene Amplicons

DNA was extracted from sediment samples using the MOBIO Power Soil DNA Extraction kit (MoBio Laboratories, Carlsbad, CA, USA). DNA concentration and quality were checked using a NanoDrop Spectrophotometer. Sediment DNA was diluted to 10 $ng \cdot \mu L^{-1}$ and stored at –40 °C for downstream use. The universal primers 515F-909R with 12 nt unique barcode were used to amplify the V4 region of the 16S rRNA gene [21,22]. The PCR reaction mixture (25 µL) contained 10 ng sediment DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.4 µM of each deoxynucleoside triphosphate, 1.0 µM of each prime, and 0.5 U Ex Taq (TaKaRa, Dalian, China). The PCR cycling profiles were as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60 s; followed by 72 °C for 10 min. Two individual PCR reactions were conducted for each sample, and the amplification products were combined and examined using 1.0% agarose gel electrophoresis. The correct-sized amplicons were excised from the gel and purified using the SanPrep DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). Then the purified products were quantified and equal molar amount from each sample were pooled together. Sequencing libraries were prepared using a TruSeq DNA kit. Then sequencing was performed using an Illumina Miseq system with the Reagent Kit v22 × 250 bp, as described by the manufacturer' instructions.

2.5. Pyrosequence Data Analysis and Putative Function Prediction

Raw sequencing data were processed using QIIME Pipeline [23]. All sequence reads were trimmed and assigned to each sample based on their barcodes. The sequences with low quality and

shorter lengths were removed, and the qualified sequences were assigned to operational taxonomic units (OTUs) at a 97% similarity level. All the samples were randomly resampled to 10,458 reads. Alpha-diversity (chao1 estimator of richness, Shannon's diversity index, and Simpson index) and beta-diversity (principal coordinates analysis (PCoA)) were calculated based on weighted UniFrac analyses. OTUs were classified taxonomically using the Ribosomal Database Project classifier. Obtained nucleotide sequences were deposited with the European Nucleotide Archive (ENA) under accession numbers ERS2573254–RS2573279. The putative ecological functions of the prokaryotic OTUs were assessed using Functional Annotation of Prokaryotic Taxa (FAPROTAX) and visualised using TBtools (v 0.665) [24].

2.6. Statistical Analyses

One-way analysis of variance (ANOVA) was used to test differences in sediment properties, relative abundance of taxonomic units, and alpha diversity among plant treatments. Significance of taxonomic differences among treatments was further examined using Linear discriminant analysis (LDA) Effect Size (LEFse) [25]. The method employs the Kruskal–Wallis sum-rank test ($\alpha = 0.015$) to identity taxa with significantly different abundances between treatments based on against all comparisons, followed by LDA to estimate the effect size of each differentially abundant taxa (LDA score > 3). The correlations between sediment properties, decreased N and the 50 most abundant microbial genera were assessed by Pearson's correlation. Redundancy analysis (RDA) were performed using the vegan package in R (R Project 3.1, v.2.3–1) to determine the environmental variable that best explained the variation of microbial community composition in plant-treated sediment.

3. Results

3.1. Nutrient Retention in Sediment

Nutrient retention in sediment samples from the MPDS system varied with different plant treatments after 150-day nurturing with rural wastewater (Table 2). NH_4^+-N concentrations in sediments were lower than 0.05 mg·kg^{-1} for all treatments. NO_3^-N concentrations were significantly lower in Tyl (0.04 mg·kg^{-1}) compared with Acg (6.09 mg·kg^{-1}) and Phh (8.64 mg·kg^{-1}). Sediment TN content increased for all treatments after nurturing with rural wastewater; the highest TN content was found in Acg (1.18 g·kg^{-1}), followed by CK, Rum, Cai, Phh, Myv, Tyl, Eic, and Ijt, ranging from 0.87–1.09 g·kg^{-1}. TP concentrations also increased for all plant treatments and were significantly higher for Ijt, Myv and Eic than for CK. However, DOC, organic C, and pH did not differ significantly among treatments.

Table 2. pH, water content and nutrients retention in sediment samples.

	pH	Water Content (%)	NH_4^+-N (mg·kg^{-1})	NO_3^--N (mg·kg^{-1})	TDN (mg·kg^{-1})	TN (g·kg^{-1})	TP (g·kg^{-1})	TK (g·kg^{-1})	DOC (mg·kg^{-1})	Organic C (g·kg^{-1})
Tyl	8.47 ± 0.16 a	50.92 ± 8.91 a	0.02 ± 0.02 ab	0.04 ± 0.06 a	2.25 ± 0.57 a	0.89 ± 0.19 ab	0.64 ± 0.04 ab	18.11 ± 1.01 ab	10.62 ± 1.27 a	13.20 ± 3.37 a
Phh	8.28 ± 0.03 a	51.25 ± 3.35	0.04 ± 0.02 b	8.64 ± 2.59 c	11.66 ± 3.00 c	0.91 ± 0.02 ab	0.63 ± 0.02 ab	16.99 ± 0.76 ab	9.72 ± 1.44 a	13.83 ± 0.76 a
Cai	8.44 ± 0.07 a	44.65 ± 2.30 a	0.05 ± 0.03 b	1.13 ± 0.49 ab	3.37 ± 1.17 ab	0.93 ± 0.10 ab	0.61 ± 0.04 ab	18.58 ± 1.60 ab	9.85 ± 1.11 a	14.25 ±1 53 a
Eic	8.55 ± 0.13 a	42.58 ± 4.23 a	0.02 ± 0.00 ab	5.70 ± 1.56 bc	9.27 ± 1.96 bc	0.89 ± 0.13 ab	0.67 ± 0.01 b	17.61 ± 2.23 ab	10.97 ± 1.08 a	14.06 ± 3.10 a
Myv	8.52 ± 0.22 a	46.01 ± 12.72 a	0.00 ± 0.00 a	2.48 ± 2.70 ab	4.67 ± 3.70 ab	0.90 ± 0.10 ab	0.68 ± 0.05 b	17.34 ± 1.27 a	10.68 ± 2.32 a	14.08 ± 0.66 a
Acg	8.33 ± 0.06 a	44.21 ± 4.79 a	0.05 ± 0.04 b	6.09 ± 5.68 bc	9.62 ± 7.10 bc	1.18 ± 0.09 b	0.61 ± 0.04 ab	20.24 ± 1.75 b	14.15 ± 10.92 a	17.14 ± 2.53 a
Rum	8.48 ± 0.22 a	46.95 ± 16.55 a	0.01 ± 0.01 a	4.89 ± 0.84 abc	8.39 ± 1.51 abc	0.97 ± 0.31 ab	0.65 ± 0.06 ab	16.99 ± 0.76 a	12.48 ± 4.37 a	15.70 ± 6.53 a
Ijt	8.57 ± 0.04 a	39.19 ± 5.78 a	0.00 ± 0.00 a	4.83 ± 2.64 abc	8.03 ± 3.17 abc	0.87 ± 0.08 a	0.68 ± 0.05 b	15.97 ± 0.04 a	13.77 ± 1.02 a	14.91 ± 2.00 a
CK	8.54 ± 0.06 a	48.55 ± 5.50 a	0.03 ± 0.01 ab	1.41 ± 1.99 ab	3.83 ± 2.41 ab	1.09 ± 0.09 ab	0.60 ± 0.01 a	18.21 ± 0.87 ab	8.84 ± 0.69 a	17.78 ± 2.68 a

Data (mean ± SD) in the same column with different small letters indicate significantly different ($p < 0.05$).

3.2. Taxon Richness and Diversity Coverage Assessment

Based on a 16S rRNA gene sequence similarity cut-off value of 97% for 271,908 reads, a total of 36,344 OTUs were observed in the 26 sediment samples. Rarefaction curves enabled the assessment of differences in species richness among sediment samples with different plant treatments (Figure 1). Sediments treated with plants had steeper rarefaction curves with higher taxon richness, compared with the CK treatment, indicating that the number of observed species was greater in sediments with plants. The Chao 1 estimator demonstrated that microbial community richness ranged from 8133 to 9467 taxa in the plant-treated systems compared with 5963 taxa in CK, which illustrated the significant impact of plants on microbial community richness (Figure 1). Moreover, the average Shannon's diversity index values of microbial communities in Ijt (11.35), Tyl (11.34), Phh (11.25), Myv (11.09), and Cai (10.75) were significantly higher than Eic (9.24) and CK (7.63) ($p < 0.05$). The Simpson diversity index demonstrated a similar pattern with the Shannon's diversity with values ranging from 0.979 to 0.999 in plant-treated sediment samples compared with 0.955 in CK.

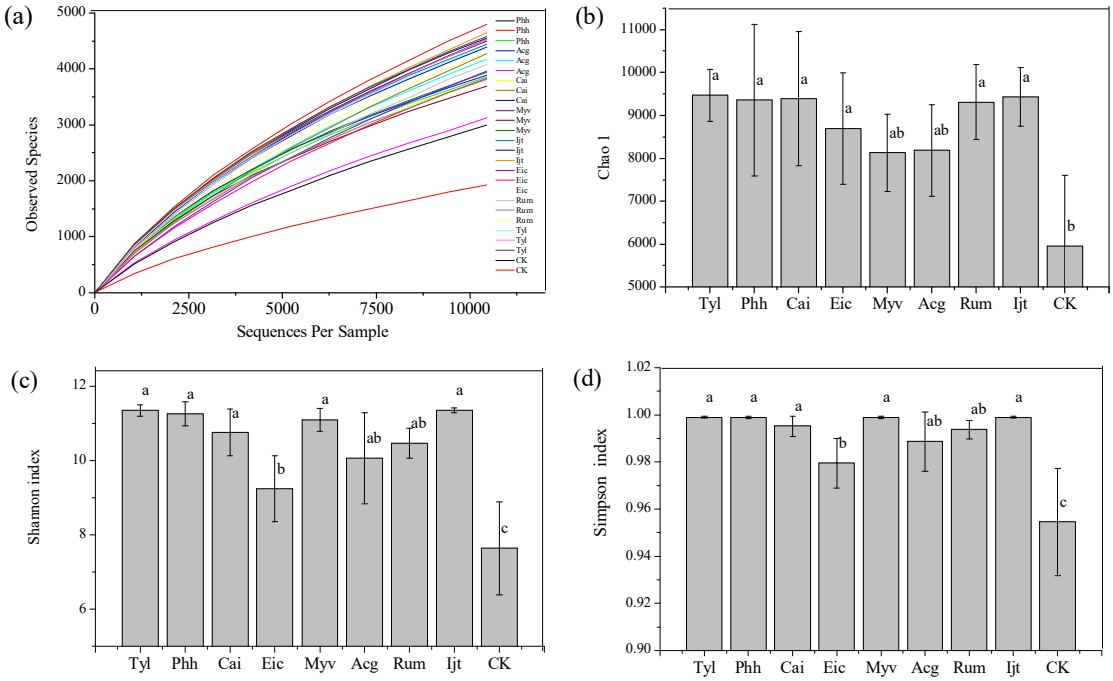

Figure 1. Comparison of Alpha diversity of sediments treated with different plant species at a phylogenetic distance of 3% (mean ± SD). (**a**) observed species, (**b**) Chao 1, (**c**) Shannon's diversity, and (**d**) Simpson index. Letters indicate significant differences. *Phyllostachys heteroclada F.* (Phh), *Acorus gramineus O.* (Acg), *Canna indica* (Cai), *Myriophyllum verticillatum* (Myv), *Iris japonica Thunb* (Ijt), *Eichhornia crassipes* (Eic), *Rumex patientia* (Rum), *Typha latifolia* L. (Tyl) and no plant (CK).

3.3. Prokaryotic Community Composition

Microbial community composition also reveals important information regarding the impact of different plants species on contaminant removal. The relative abundance of prokaryotic 16S rRNA gene in the sediment samples at phylum level is shown in Figure 2. A total of 77 different phyla were detected in the 26 sediment samples. *Proteobacteria*, *Crenarchaeota*, *Chloroflexi*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Euryarchaeota*, *Nitrospira*, *Firmicutes*, *[parvarchaeota]*, *Spirochaetes*, *Gemmatimonadetes*, *WS3*, and *Chlorobi* were the 15 most abundant phyla (in descending order of abundance). A total of 529 orders were detected, of which 110 orders were shared by all samples, and among which 10 orders were abundant (>1%) in all plant-treated samples, including *Bacteroidales*, *Xanthomonadales*, *Syntrophobacterales*, *Rhizobiales*, *envOPS12*, *Burkholderiales*, *Rhodospirillales*, *Myxococcales*, *Bacteria* (no rank), and *iii1-15*. At the family level, 847 families were detected and

137 families were shared by all samples. Twenty-one abundant families were detected (average abundance of all samples >1%), including *SAGMA-X*, *WCHD3-30* (no rank), *Bacteria* (no rank), *iii1-15*(no rank), *Koribacteraceae*, *Ellin6513*(no rank), *Bacteroidales* (no rank), *Chitinophagaceae*, Anaerolinaceae, *GCA004* (no rank), *envOPS12* (no rank), *Hyphomicrobiaceae, Rhodospirillaceae*, *Betaproteobacteria*(other), *Betaproteobacteria* (no rank), *MND1*(no rank), *Rhodocyclaceae*, *Syntrophaceae*, *Syntrophobacteraceae*, *Sinobacteraceae*, and *Spirochaetaceae*. At the genus level, 1426 genera were identified, among which 147 genera were shared by all 26 samples, accounting for 59.2% of observed OTUs. The top 50 abundant genera in total classified sequences were selected and their abundances compared, as shown in Figure S1.

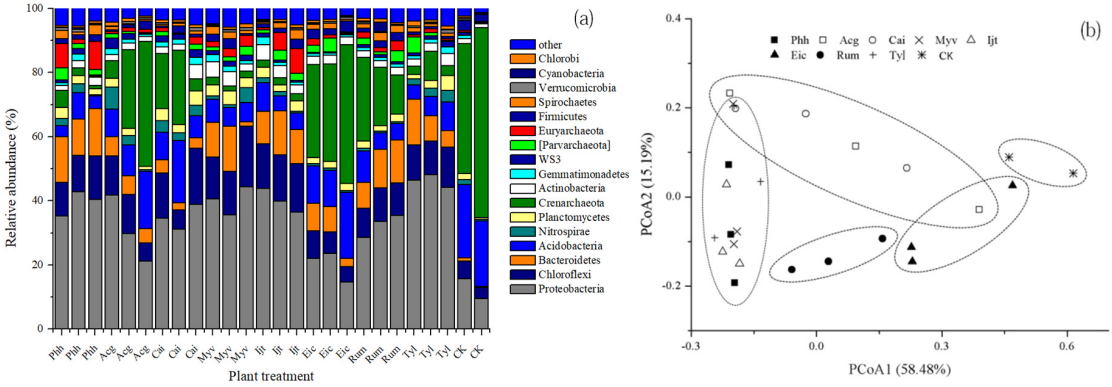

Figure 2. Relative abundance of prokaryotic 16S rRNA gene in the sediment samples at phylum level (**a**); PCoA of sediment prokaryotic community based on the weighted UniFrac distances (**b**). Circles indicate the differentiation between plant treatments.

The difference in relative abundance of dominant prokaryotic taxa among plant treatments was determined by ANOVA. *Crenarchaeota* was the predominant phylum in CK and Eic, accounting for 49.85% and 34.15% of the total phyla, respectively, demonstrating less abundance in Acg, Rum and Cai. *Crenarchaeota* was significantly less abundant in Tyl, Phh, Ijt, and Myv, ranging from 1.97–4.88% of the total sequence ($p < 0.05$). The dominant genera from the phylum *Crenarchaeota* included *SAGMA-X* (no rank), *Nitrosotalea*, *Candidatus Nitrososphaera* (family *Nitrososphaeraceae*), and *pGrfC26* (no rank). The relative abundance of *Nitrosotalea* (family *SAGMA-X*) accounted for 3.2% of all genera in CK, followed by 2.27% in Eic; 1.32% in Acg; and 1.19% in Rum; with <1% in Phh, Ijt and Myv. The genus *Candidatus Nitrososphaera* exhibited significantly lower abundance in CK (0.30%) than that in Cai (1.48%). *Acidobacteria* comprised 21.59% in CK and was significantly decreased to 14.57% in Ei, 11.97% in Acg,11.53% in Cai, and 5.17–6.72% in other treatments ($p < 0.05$). *Proteobacteria* was the third-most abundant phylum in CK (accounting for 12.69%) and the most prevalent phylum in vegetated groups (30.93–46.26%) except for Eic (20.12%). *Beta-* and *Deltaproteobacteria* were the most abundant subdivisions of *Proteobacteria*, followed by *Gamma-* and *Alphaproteobacteria*. The relative abundance of *Betaproteobacteria* was highest in Tyl (17.89%), followed by Phh (12.31%), Ijt (11.95%), Cai (11.40%), Acg (11.21%), Myv (11.12%), Rum (9.13%), Eic (4.88%), and CK (3.76%). Eighteen taxa were identified within the *Betaproteobacteria*, among which *Burkholderiales* was the dominant shared class in all samples, with the highest abundance in Tyl (3.41%), Ijt (3.09%), Phh (2.84%), and Rum (2.43%), and the lowest abundance in CK (0.82%). Fifteen abundant genera of *Betaproteobacteria* were shared in all samples, including two genera from *Comamonadaceae* and three genera from *Rhodocyclaceae*. *Methylococcales* was a notable order within *Gammaproteobacteria*, which was significantly more abundant in Tyl (0.82%) and Phh (0.56%) than CK (0.04%). Significantly lower abundance of *Chloroflexi* was detected in CK (4.48%) compared with the vegetated treatments (6.74–15.09%). The highest abundance of *Bacteroidetes* was detected in Phh (13.45%), followed by Ijt (11.50%), Rum (11.22%), Tyl (9.14%), Myv (8.77%), Eic (6.29%), Acg (5.45%), Cai (3.32%), and CK (0.67%). *Actinobacteria* abundance was significantly higher in Myv, Ijt and Tyl with average values of 3.81%, 3.76% and 2.84% compared with

CK (1.39%). The abundances of *Nitrospirae*, *WS3*, and *Gemmtimonadetes* were remarkably higher in Cai, Acg, Myv, and Tyl than in CK. Highest abundance of *Spirochaetes* was identified in Rum (1.97%), while the abundance in CK was only 0.12%. In addition to *Crenarchaeota* and *Acidobacteria*, *Firmicutes* was another dominant phylum with high abundance in CK (2.62%) compared with most of the vegetated treatments (1.19–1.93%).

PCoA based on weighted Unifrac distances was conducted to statistically assess the similarity of prokaryotic communities in different plant-treated sediments. The results of PCoA analysis with maximum variation of 58.48% (PC1) and 15.19% (PC2) are shown in Figure 2b. PCoA analysis revealed that prokaryotic communities were separated by plant treatment. Phh, Myv, Tyl, and Ijt were separated from other treatments on the PCoA 1 axis.

3.4. Abundance Differences Among Sediments Treated with Different Plant Species

The differentially abundant features among sediments treated with different plant species were identified by LEfSe analysis (Figure 3). A total of 58 taxa presented significant differences from five plant treatments with a LDA threshold of 3. The significantly differential abundant taxa in CK belonged to the phylum *Crenarchaeota*. The most differentially abundant taxa in Tyl belonged to the order *Rhodocyclales* and the family *Alcaligenaceae*, followed by *SC_|_84*, *SBla14*, *Methylococcales*, *Alphaproteobacteria*, *Methylibium*, *Dechloromonas* (family *Rhodocyclaceae)*, *Opitutaceae*, *Opitutae*, *Opitutales*, *TSCOR003_O20*, *Fibrobacteres,* and *WS2*. *Euryarchaeota and Methaomicrobia* presented the highest LDA score in Phh, followed by *125ds10*, *Methanomassiliicoccacceae*, *Syntrophorhabdaceae*, *Dwsulfobulbus*, *MBGB*, *Crenothrix*, *Crenotrichaceae*, *Leptothrix, and Methanobacteriaceae*. Twenty clades including *Xanthomonadales*, *Anaerolineales*, *Sinobacteraceae* (order *Xanthomonadales)*, *Spirochaetes*, *Spirochaetales*, *Steroidobacter*, *Syntrophaceae*, *Ellin6529*, *Thiotrichales*, *Piscirickettsiaceae*, *Planctomycetaceae*, *Planctomyces*, *Planctomycetales*, *Nannocystaceae*, *Myxococcaceae*, *Pirellula*, *BRC1*, *Nannocystis*, *Adhaeribacter,* and *NKB19* showed abundance advantage in Myv treatment. *Anaerolinea*, *Desulfobacterales*, *Ellin6067*, *Pirellulaceae*, *Desulfobulbaceae*, *Comamonadaceae*, *Thermoplasmata*, *YLA114*, *Burkholderiales*, *Gaiellales*, and *Syntrophobacter* were present in high proportions in Ijt.

3.5. Effect of Plant Species on Putative Functions of Sediment Prokaryotic Communities

A total of 4439 OTUs (out of 36,343 OTUs) were classified into 91 specific functional categories according to the FAPROTAX prediction. The significant correlation between predicted functional composition and taxonomic composition (Mantel $r = 0.301$, $p = 0.003$) indicated the prediction is significative for all samples. The functional capacity of N metabolism was significantly affected by plant species, including N fixation, nitrification, and denitrification (Figure 4). The N fixation process was enhanced by Ijt treatment ($p < 0.05$) and positively correlated with NO_3^--N ($r = 0.484$, $p < 0.05$, Table S2). The predicted nitrification was reduced in Ijt, Phh, Rum, and Myv ($p < 0.05$) and demonstrated positive correlations with TN ($r = 0.577$, $p < 0.01$) and NH_4^+-N ($r = 0.301$, $p = 0.136$). Further, anaerobic ammonium oxidation (anammox) was also positively related to NH_4^+-N ($r = 0.552$, $p < 0.01$). Denitrification and nitrite ammonification were positively related to NO_3^--N ($r = 0.135$, $p = 0.547$; $r = 0.445$, $p < 0.05$, Table S2) with the lowest values in Cai.

3.6. Correlations between Environmental Variables and Microbial Composition

RDA was performed to correlate the relative abundance of prokaryotic community (the top 50 abundant genera in total classified sequences were selected) with sediment parameters to determine the major environmental variables that impact the microbial community structure. As shown in Figure 5, this model attributed 52.9% of variance to species-environment correlations. The first and second axes represented 20.5% and 17.9% of variation, respectively. NH_4^+-N and TP were significant variables ($p < 0.01$), suggesting they were major environmental factors influencing the microbial community structure. Microbial community in CK, Acg, Cai, and Tyl were primarily linked to NH_4^+-N and TN, while those in Ijt, Myv and Phh treatments were associated with TP. The abundances of

SAGMA-X (no rank), *Nitrosotalea* (family *SAGMA-X*), *Ellin6513* (*Acidobacteria*), and *Koribacteraceae* (*Acidobacteriales*) presented strong positive correlations with NH_4^+-N and SOC, while the abundances of *Bacteroidales* (no rank), *Cytophagaceae* (*Bacteroidetes*), *Anaerolinaceae* (*Chloroflexi*), *Rhodoplanes* (*Rhizobiales*), *Gemm-1*(*Gemmatimonadetes*), Geobacter(*Desulfuromonadales*), *Treponema* (*Spirochaetaceae*), *Methanosaeta* (*Methanosaetaceae*), *Syntrophaceae*, *Steroidobacter* (*Xanthomonadales*), and *Candidatus Methanoregula* (*Methanoregulaceae*) demonstrated positive correlation with TP and negative correlations with SOC and TN.

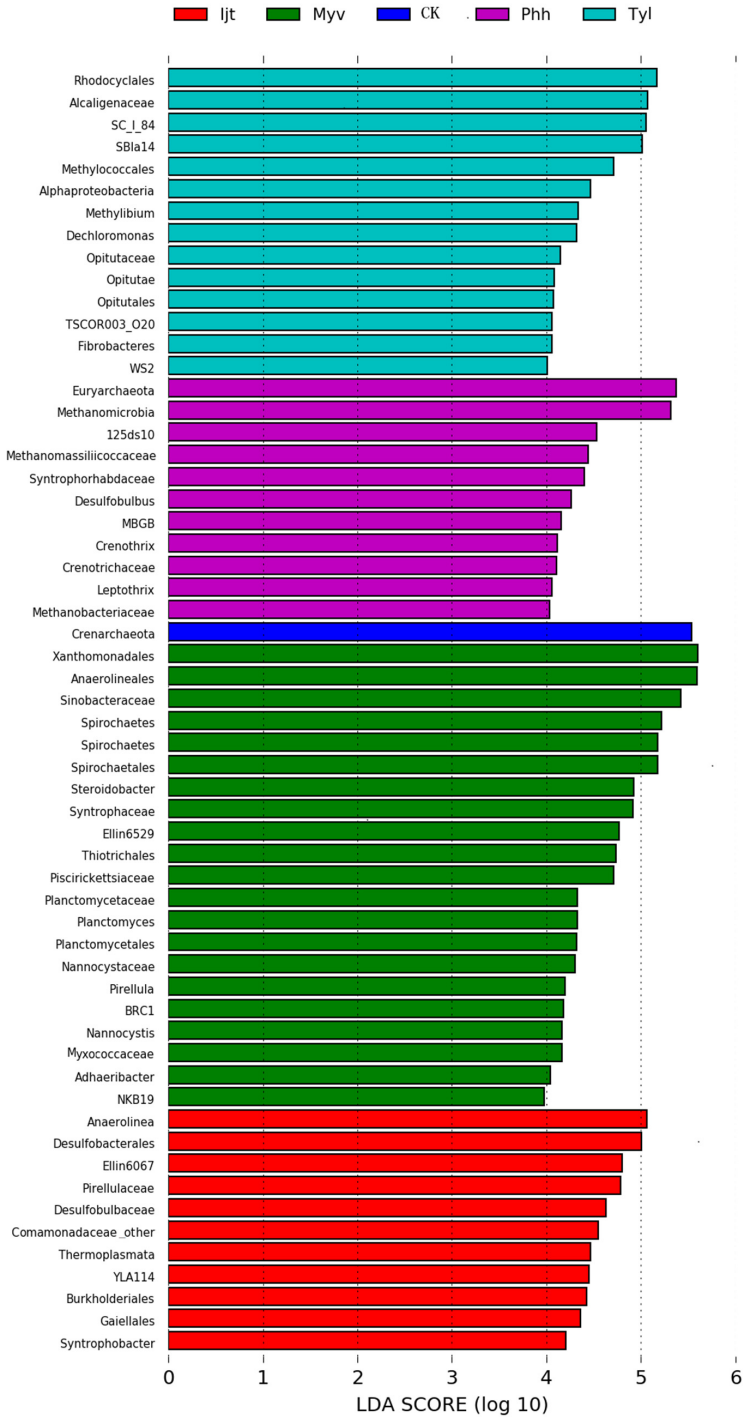

Figure 3. LEfSe analysis of microbial abundance in sediments with different plant species. LDA score represented the differentiation size among plant treatments with a threshold value of 3.

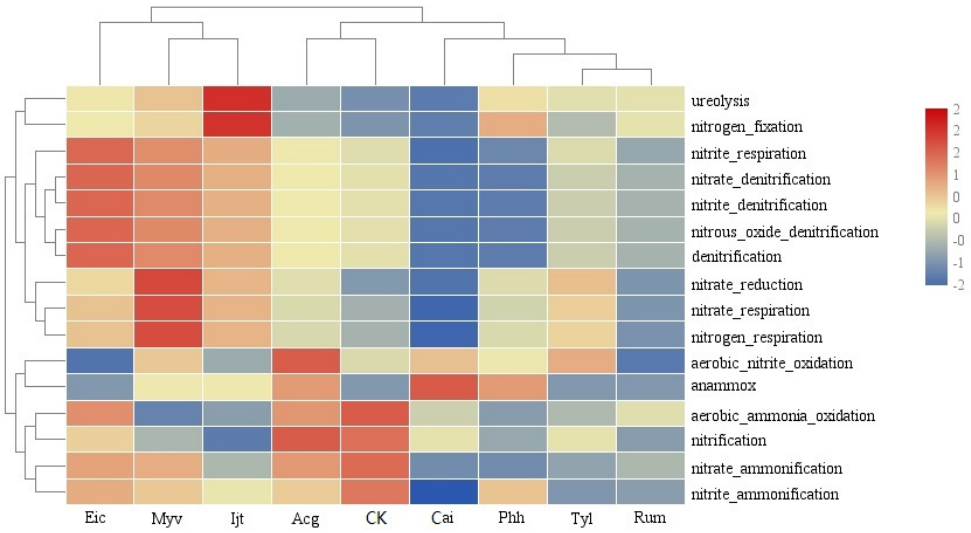

Figure 4. Predicted functional profiles associated with N cycling under different plant treatments.

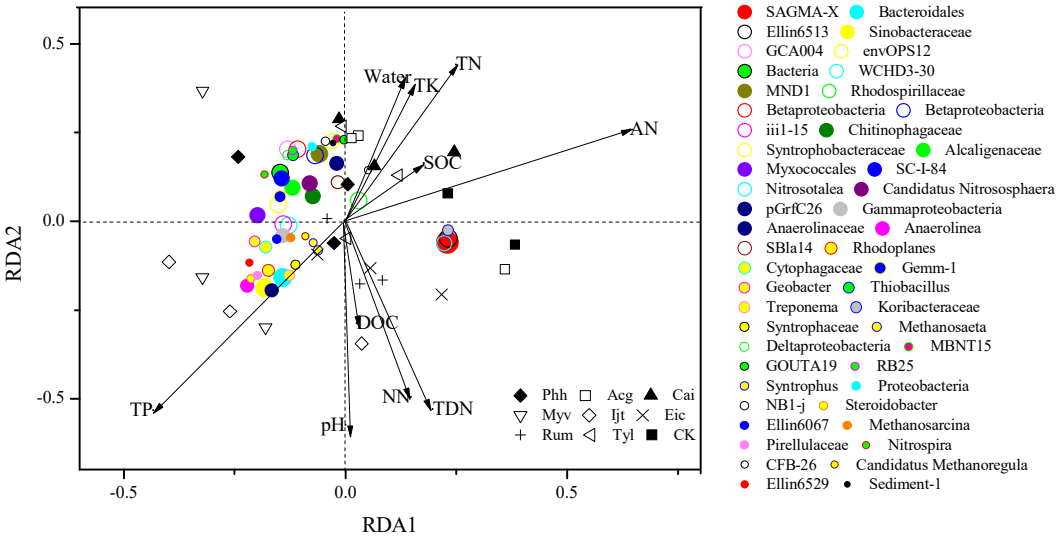

Figure 5. RDA analysis of the relative abundance of prokaryotes (genus level) and measurable variables in the 26 sediment samples. AN: NH_4^+-N; NN: NO_3^--N; Water: Water content.

4. Discussion

Eco-ditches have been successfully employed to treat rural contaminants [26,27]. Plants, one of the most vital components for N uptaking from the eco-ditch system, provide large surface areas for microbial colonisation that improve the removal ability of the rhizosphere [9]. Our previous study suggested Tyl, Myv, and Rum had the highest efficiency for N accumulation, followed by Eic, Phh, Cai, Acg, and Ijt [9]. The results of the current study demonstrated that N retention in sediments of MPDS systems increased after 150-day treatment with wastewater, exhibiting 90–106% increases in Acg and CK, with 51–68% increases observed in other plant treatments (Table 2 and Table S1). These results indicated that the N retention pathway in sediment was significantly impacted by plant species. Different plant species vary in radial oxygen loss, root exudates, and their subsequent impact on the sediment environment. The specific microbial populations of different kinds of plants are associated with specific root exudates and rhizodepositions [28]. Results of 16S rRNA gene pyrosequencing demonstrated that sediment microbial abundance was significantly impacted by plant species (Figures 2 and 3). Thus, the difference in sediment N retention among plant treatments was linked to microbial community structure in the sediment, as expected.

Conventional nitrogen removal from wastewater is known to comprise two key microbial processes: nitrification and denitrification. Nitrification is the microbially-mediated oxidation of ammonia (NH_3) to nitrite (NO_2^-) and nitrate (NO_3^-), which may ultimately be removed from the system by reduction to dinitrogen by denitrification or anammox. The rate-limiting step in the nitrification process is the oxidation of NH_3 to NO_2^-, which is performed by AOB including only a few special genera, such as *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* [29]. In this study, extremely small proportions of these three genera were detected in all samples. However, we found large proportions of *Crenarchaeota* in CK, Acg and Cai, which includes the widely existing ammonium-oxidising archaea (AOA) and is implicated in the process of oxidising ammonia to nitrite. Quantitive PCR of gene copies in a local eco-ditch sediment also showed that the absolute abundance of archaeal *amoA* was 10–100 fold higher than bacterial *amoA* genes (Figure S2). *Nitrosotalea* (from *Crenarchaeota*) abundance demonstrated positive correlations with sediment NH_4^+-N contents (Figure 5) and ammonia oxidation function predicted by Faprotax ($r = 0.860$, $p = 0.000$), indicating that *Nitrosotalea* might be the dominant ammonium-oxidising microbes in sediment samples. Previous studies reported that *Nitrososphaera* was the dominant AOA genus in natural freshwater wetland [30,31]. In the present study, *Candidatus Nitrososphaera* occupied 0.52–1.48% for planted treatments and 0.30% for CK. Plant root exudes organic carbon compounds, which are essential for the growth of *Nitrososphaera* and could be conductive to the high proportion of *Nitrososphaera* [32]. *Nitrospira* is generally considered to be the major contributor for oxidation of nitrite to nitrate and occupied 0.56–0.70% of all detected sequences in Myv, Cai, and Tyl, which was significantly higher than other treatments. *Nitrospira* abundance was positively related to nitrite oxidation predicted by Faprotax ($r = 0.541$, $p < 0.01$) and negatively related to NO_3^-N ($r = -0.451$, $p < 0.05$). We also found a positive relationship between NH_4^+-N and anammox ($r = 0.552$, $p < 0.01$). Therefore, the key contributors for NH_4^+-N removal from wastewater sediment probably include ammonia-oxidising archaea, nitrifying bacteria and anammox.

Denitrification is the reduction process from nitrification product(s) to gaseous nitrogen compounds (mainly dinitrogen) and is widely used in bioecological wastewater treatment to remove NO_3^--N. Especially in the anoxic sediments of aquatic systems, denitrifying bacteria play the most significant part in long-term N removal [33]. The abundances of the core families responsible for denitrifying processes, *Rhodocyclaceae* (*Rhodocyclales*) and *Comamonadaceae* (*Burkholderiales*) [34,35], varied significantly with different plant treatments. The order *Rhodocyclales* exhibited the highest score in Tyl treatment, in which the genus *Dechloromonas* (*Rhodocyclaceae*) was also predominant (Figure 3). *Dechloromonas* is linked to denitrification and is frequently found in wastewater treatment plants. *Thaurea,* another important denitrifying genus in the family *Rhodocyclaceae*, exhibited significantly higher abundance in *Tyl* (0.21%) than in Eic, Myv, and CK (0.03–0.06%) ($p < 0.05$). Higher gene copies of denitrifiers (*nosZ*) were also detected in Tyl than that in Phh- and Cai-treated ditch sediment using quantitive PCR (Figure S2). The family *Comamonadaceae* also comprises anaerobic denitrifiers [36]. A dominant genera of *Comamonadaceae* (no rank) found in Ijt is reportedly capable of denitrification [37,38]. A previous study reported that *DA052* (affiliated with *Acidobacteria*) had the potential to utilise oxidised N, such as NO_2^- and NO_3^-, as additional N sources, to carry out assimilatory sulfate reduction [39]. This study reported that *DA052* abundance was higher in CK (12.84%) and Eic (7.87%) than other plant treatments (0.01–4.48%). Plant treatments improved the O_2 content in sediment and the survival of *Bacteroidetes*, which exhibited higher abundance in plant- treated sediments (especially Phh, Ijt, Rum, Tyl, and Myv) compared with CK (Figure 2, $p < 0.05$). Members of *Flavobacterium* from *Bacteroidetes* have been reported to be capable of denitrification [40–43]. In summary, unlike the autotrophic nitrifying bacteria responsible for nitrification, denitrifying bacteria are composed of ubiquitous, heterotrophic organisms [44]. The low NO_3^--N content in Tyl and CK might be associated with denitrifier differentiation, including heterotrophic denitrifiers, aerobic denitrifiers, facultative autotrophic bacteria, etc. However, the predicted denitrification was not significantly associated with NO_3^--N content ($r = 0.135$, $p = 0.547$). It should be noted the FAPROTAX prediction is based on cultured strains and these results warrant further verification.

5. Conclusions

In this study, we analysed the prokaryotic community composition in sediments from MPDS systems treated with different plant species. The results demonstrated that N retention in sediments, as well as microbial richness and diversity, were significantly affected by plant species. A total of 58 differentially abundant taxa were identified by LEfSe analysis. This study indicated that specific functional microbial differentiation among plant treatments was associated with removal of certain N contaminants from the sediment of eco-ditches. The probable NH_4^+-N removal pathway in wastewater sediment was through a combination of AOA, nitrifying bacteria, and anaerobic ammonium oxidation. Additionally, NH_4^+-N and TP concentrations were identified as major environmental factors influencing microbial community structure, according to RDA. Ijt treatment significantly enhanced the ability of the microbial community to remove N contaminants from the sediment.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/11/3035/s1. Table S1: Amount and percentage of increased TN in sediment during the 150-day experiment in MPDS systems treated with different plant types. Table S2: Correlations between soil chemical properties and N cycling processes predicted by FAPROTAX.

Author Contributions: Conceptualization, Z.D. and B.Z.; methodology, Z.D., L.H. and M.N.K.; software, J.L. and J.T.; writing—original draft preparation; funding acquisition, B.Z. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded by the National Key Research & Development Program, grant Number 2017YFD0200105); the National Natural Science Foundation of China, grant Number 41301266; the CAS Western light plan and Key science and technology project in Sichuan Province, grant Number,2018SZDZX0025 and 2018SZDZX0027.

Conflicts of Interest: The authors declare no conflict of interest.

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