Effects of Waterlogging on Soybean Rhizosphere Microbial Community Profiled Using Illumina MiSeq, LoopSeq, and PacBio 16S rRNA Genes Sequences

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Research

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Abstract

Background: Waterlogging on the global environment has led to a significant decline in crop yields. However, the response of plant-associated microbes to waterlogging stress on different soils is not known. Moreover, there are few reports on whether this response is influenced by different sequencing methods. In this study, the effects of waterlogging on soybean rhizosphere microbial structure on two types of soil were examined, using a short reading 16S rRNA sequencing variable region V4 and two full-length 16S rRNA sequencing variable regions V1-V9.

Results: The results revealed some similarities and differences in three sequencing methods for soybean rhizosphere microbial response to waterlogging stress. Based on CPCoA analysis, all the sequencing methods showed that waterlogging on both types of soil significantly affected the bacterial community structure of the soybean rhizosphere, and increased the relative abundance of *Geobacter*. However, the full-length sequencing methods had higher classification resolution than short-read sequencing (except phylum level of all sequencing methods and class level of LoopSeq sequencing). Further, analysis on OTU level and network showed that waterlogging increased the abundance of some microorganisms related to nitrogen cycle using V4 sequencing, and microorganisms related to phosphorus cycling when using two full-length sequencing methods. This is in line with the core microbial analysis. Environmental factors affecting the structure of microbial communities differed among sequencing methods.

Conclusions: In summary, this piece of work detected the effects of waterlogging on soybean rhizosphere microbes using three sequencing methods. Some functional microbes were enriched in the rhizosphere, which may benefit soybean in resisting waterlogging stress. On the other hand, there were several differences in results among the three sequencing methods which might affect the response of rhizosphere microbial structure to stress. Our analysis of sequencing methods on various levels provides some useful information on environmental samples sequencing.

Introduction

A significant decline in crop yields has been witnessed globally as a result of drought, waterlogging and extreme temperatures in the past few decades [1, 2]. Waterlogging has been projected as one of the abiotic stresses that will adversely affect the physiological growth of plants [3–6]. In particular, soil physicochemical properties (e.g., porosity, structure, and pH) and activity of microbial communities will be the worst affected [7, 8]. The loss of nitrogen (N) in waterlogged soil is expected, which in combination with other harmful effects (such as root hypoxia), will lead to the reduction of crop productivity [9]. Therefore, collective efforts are needed to reduce the adverse effects of waterlogging stress on crop production [10]. Even though much has been achieved by the genetic improvement of crop cultivars and cultivation measures that mitigate waterlogging, the role played by rhizosphere microorganisms in plant resistance to waterlogging has been limitedly studied.

Some microorganisms regulate the growth of plants either by resisting or causing stress [11]. Several studies have reported microorganisms with varied positive effects growing near stressed plants [12, 13]. For example, beneficial bacteria containing 1-aminocyclopropane-1-carboxylic acid deaminase can reduce stress-induced ethylene content, thereby protecting plants from waterlogging [14, 15]. Moreover, some bacteria synthesize indole-3-acetic acid in the rhizosphere [16], which helps in alleviating water stress [17]. An anaerobic condition caused by waterlogging stress might change the structure of the soil microbial community [18]. Because the microbial community is involved in the nutrient cycle, changes in their structure might further affect the composition of the ecosystem [19]. Therefore, prospecting microorganisms that are beneficial to plant adaptation under waterlogging stress is a viable option to mitigate waterlogging [20].

High-throughput sequencing of partial 16S rRNA is the most common method used to analyze the microbial community due to its low cost [21]. Often only V1-V3, V3-V5, or V4 of the nine variable regions in the 16S rRNA gene (called V1-V9) have been interrogated [22–24]. However, the taxonomic classification, as well as the abundance of and diversity operational taxonomic unit (OTU), are affected by variable region selection [25, 26]. Moreover, the accuracy and sensitivity of taxonomic discrimination and estimates of taxon abundance are significantly influenced by sequence read length and primer selection [27, 28]. The short-read approach by second-generation sequencing was found to be affected by the variable region biasness and could not provide valid information beyond the genus level. This resulted in an inaccurate classification of sequences of environmental samples [29, 30]. Low-resolution classification not only limits the accuracy of microbial ecological function inference and host metabolic reconstruction but also affects the appropriate identification of bacterial strains in subsequent experiments and transformation studies [30].

Pacific Bioscience (PacBio) has developed a long-read sequencing technology that can complete full-length 16S rRNA genes (V1-V9) sequencing at comparatively high throughput. Moreover, the initial high intrinsic error rate has been improved by the circularized library templates combined with highly processed polymerases that allow for the “circular consensus sequence” (CCS) read with sufficiently high quality [31]. However, few studies have used environmental samples to analyze the performance of the PacBio platform under full-length 16SrRNA sequencing [32, 33].

Another full-length sequencing platform is LoopSeq, a single-molecule counting technology that is way cheaper than PacBio. It can eliminate biasness caused by PCR and can sequence the molecules with very low abundance. In this process, qPCR is performed to first quantified the DNA before pooling the DNA into a single reaction. The sequencing procedure lowers the effects of microbial absolute abundance among different samples, and therefore, a more accurate picture of the species in the sample is reported. Moreover, each 16S molecule is barcoded before clustering and assembling into a single long-read. This results in overlapping of each base position with multiple short-read sequences and allows for consensus calling to determine the true call independent of sequencing errors (< 0.005%) [34].

In the current study, the effects of waterlogging stress on soybean rhizosphere microbial structure on two types of soil were explored using the partial 16S rRNA by Illumina MiSeq platform and full-length 16S gene sequencing by LoopSeq and PacBio platform. We hypothesized that (i) the waterlogging can changed the microbial community structure and diversity of the two soils, (ii) the resolution of full-length sequencing was higher than that of partial sequencing, and (iii) the full-length sequencing by LoopSeq and PacBio provided more accurate information on the screening of critical bacterial species in resistant to waterlogging stress than partial 16S rRNA gene sequencing.

Methods And Material

Soil and soybean material

A neutral and an acid soil were collected from Yingde County (113°40'N, 24°18'E), and Suixi County (110°25'N, 21°32'E), Guangdong Province, China, respectively. Two soybean (*Glycine max* L.) varieties with different waterlogging tolerance, i.e., Qihuang34 (tolerant) and Jidou17 (sensitive), were used in this study [35, 36].

Experimental design

We conducted a random pot experiment in the greenhouse in the Agricultural College of South China Agricultural University, Guangzhou, China. Each treatment on each cultivar was done in six repetitions. The air-dried soil was sifted through a 2 mm sieve to remove impurities before planting the soybean. Each pot (top diameter 13.8 cm, bottom diameter 10.4 cm, height 12.2 cm) used in this study contained about 2.5 kg of air-dried soil. Eight strong and full soybean seeds with similar shapes were sown in each pot. The soybean growth process was carried out in a greenhouse with controllable conditions (temperatures of 26–32°C in the daytime and 15–21°C in the nighttime). After 6 days of emergence, the seedlings were removed from the 3 soybean plants. Waterlogging stress examination was performed at the soybean in the V2 stage. The water was added to the pots up to 4 to 6 cm above the soil for the experimental cultivars, while the control plants were left in an ideal environment.

Soil sampling

After three days of waterlogging treatment, the rhizosphere soil of soybean was collected and the roots transferred into a 50 ml centrifuge tube filled with phosphate-buffered saline (PBS). After centrifugation for 10 minutes, 5 g of deposited rhizosphere soil was collected from each sample and placed in a sterilization centrifuge tube for storage at -80 °C for DNA extraction. The remaining rhizosphere soil was stored at 4°C prior to the determination of soil physical and chemical properties.

Analysis of soil properties

Soil pH was determined using a pH meter (FE20-FiveEasy™ pH, Mettler Toledo, Germany) in soil water suspension (5:1 water-to-soil). Total nitrogen (TN) was determined using an Ultraviolet Spectrophotometer (UV-1800, Suzhou, China). Total potassium (TK) in soil was measured by flame

atomic absorption spectrometer (AA-7000, Shimadzu, Japan). Soil organic carbon (SOC) content was assessed using a TOC-5000A analyzer (Shimadzu, Kyoto, Japan). The content of NH_4^+ , NO_3^- , TP, and Olsen-P in soil was determined by a continuous flow analytical system (SKALAR SAN⁺⁺, Netherlands).

DNA extraction from soil samples and sequencing process

Total soil DNA was extracted using a Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) following the manufacturer's recommendations. The DNA was eluted with 80 μL H_2O , and analyzed by Nanodrop 2000 spectrophotometry. Primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTCTTAAT-3') with variable 12 bp barcode sequences were used to amplify the V4 region of the 16S rRNA gene [37]. Primers 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTACGACTT-3') were used for the full-length (V1-V9) 16S rRNA gene amplification (LoopSeq and PacBio sequence) [38]. The qPCR reaction system included 22.5 μL PCR SuperMix, 1.0 μL positive primer and 1.0 μL reverse primer, 10 ng template DNA, dd H_2O supplement to 25 μL . The amplification program was 1 cycle of 95°C for 60 s, 28 cycles of 95°C for 60 s, annealing at 58°C for 60 s and primer extension at 72°C for 2 min, and finally 1 cycle of 72°C for 10 min. Both V4 and full-length sequencing were performed according to HUADA's standard procedures. Illumina Miseq platform was used to sequence the V4 amplicon (reagent kit v.3; Illumina). The full-length sequencing of PacBio was completed on the Pacbio RS II platform.

Loopseq-16s-microorganism-24-plex-kit (Loop Genomics, San Jose, CA, USA) was used to analyze the microbial genome of rhizosphere soil. The unique molecular markers of a single 16S gene used in LoopSeq sequencing were distributed in the whole gene, and then the full-length 16S gene was recombined through short reading and sequencing on the Illumina platform. Briefly, 10ng DNA from different rhizosphere soil samples was used to build a sequencing library. The raw data were collected by Illumina's NextSeq, with generated FASTQ file [39].

Data analysis

For the V4 16S sequence, the raw FASTQ sequence file was processed by USEARCH10 [40]. After the chimeras were removed, the classification of clusters was done by comparing them with the SILVA database (Ref 119, 8 December 2014). In addition, each OTU was assigned the taxonomies at 97% cutoff using the RDP Classifier and CD-HIT [41]. For the PacBio 16S sequence, the raw sequence files were processed using SMRT Link software version 5.1.0.26412 (Pacific Biosciences). The OTUs were clustered using the UPARSE algorithm [40], and parameters were used to tune the full-length sequencing. The OTUs were iteratively classified according to the latest non-redundant small subunit SILVA using the RDP-classifier at 97% cutoff. For the LoopSeq, QIIME 2 was used to process the FASTQ files. In brief, the Divisive Amplicon Denoising Algorithm 2 (DADA2) in QIIME 2 plugin was used to obtain OTUs, which detected and corrected amplicon errors and filtered out the potential base error and chimeric sequences [42, 43]. All the raw sequences were filtered, trimmed, and dereplicated. The representative sequence generated after denoising was based on sklearn's Naive Bayes classifier for bacterial classification on the SILVA 16S full-length database [44].

Statistics analyses

Using the “vegan” package in R V3.6.3, constrained principal-coordinate analysis (CPCoA) based on the UniFrac distances derived from the phylogenetic tree, adonis test (PERMANOVA), and the mantel test were performed (Anderson, 2001). Genstat V13 was used to perform a three-way analysis of variance (ANOVA) to identify significant differences in classification resolution, soil chemical properties, bacterial alpha diversity, and the relative abundances of bacterial phyla and genera among all treatments [45]. In each sequencing method, the relative abundance of OTUs in different treatments was determined using the “DESeq2” package with the Benjamini-Hochberg correction (DESeq2, $n = 6$, $P < 0.05$). Origin was used for the visualization of bar graphs of the classification resolution of different sequencing methods. A Venn diagram showing the numbers of unique and shared microbial species on different taxonomy levels of different treatments, with the three sequencing methods was drawn [46].

The core bacteria, which contain a list of OTUs observed in 60% of V4, LoopSeq, and PacBio were obtained using Microbiome Analyst [47]. Additionally, we constructed three co-occurrence networks to analyze the correlations between OTUs in different sequencing methods (Sequence number > 5). The “psych” package in R was used to calculate Spearman’s rank correlation and P -value, and the Gephi was used for visualization [48]. Nodes were colored according to different modules. It is generally considered that nodes with a high degree, closeness centrality, and betweenness centrality values are the key species [49]. To identify the OTUs that were significantly different for the network module between neutral soils and acidic soils as well as rhizosphere soil with and without waterlogging, we established a generalized linear model of the negative binomial distribution for differential OTU relative abundance analyses [41]. Spearman correlation in R V3.6.3 was used to analyze the relationship between various environmental factors [50].

Results

The advantages and disadvantages of the three sequencing methods are presented in Table 1. In general, full-length sequencing had high-throughput nature, which could eliminate PCR biasness, and cover more sequencing areas, while V4 sequencing was the cheapest method (Table 1).

Table 1
Related attributes of different sequencing platforms

	V4	LoopSeq	PacBio
Cloning required	No	No	No
Average sequence time	8h	2.5h	2 h/SMRT cell
Average read length	~ 250bp	~ 1500bp	1000-1500bp
Read technology	Short-read technology	Long-reads technology	Long-reads technology
Sequencing variable region	V4	V1-V9	V1-V9
Stitching during sequencing	Yes	No	No
Error rates	high	lower	medium
Eliminate PCR bias	NO	Yes	Yes
Need stitching	Yes	NO	NO
Approximate cost per Mb	US\$0.11	US\$0.245	US\$2.50

Effects of waterlogging and soil types on bacterial diversity and community structure

The results from three sequencing methods showed that waterlogging had no significant impact on the soybean rhizosphere bacterial diversity, except in the samples from acidic soil sequenced by LoopSeq (one-way ANOVA, $n = 6$, $P > 0.05$; Fig. 1A). Using the V4 sequencing method, we revealed that neutral soil had higher Shannon diversities than acidic soil (one-way ANOVA, $n = 6$, $P < 0.05$; Fig. 1B). Regarding beta diversity, CPGCoA analysis revealed that waterlogging and soil types had significant effects on rhizosphere microbial community structure in the V4 and LoopSeq sequencing. However, from PacBio sequencing, only waterlogging had a significant effect on rhizosphere microbial community structure (PERMANOVA, $n = 6$, $P < 0.05$) (Fig. 1C and E, Table 2).

Table 2
Effects of waterlogging and soil types on bacterial community structure in soybean rhizosphere analyzed by permutational multivariate analysis of variance (PERMANOVA).

Sequencing methods	Factor	F	R ²	P
V ₄	Soil	111.7140	0.66882	0.001 ***
	Water	10.2152	0.06116	0.002 **
	Soil:Water	1.1018	0.00660	0.246
	NeW Vs NeCK	7.0589	0.24292	0.001 ***
	AcW Vs AcCK	6.7174	0.23391	0.001 ***
LooSeq	Soil	17.5165	0.26413	0.001 ***
	Water	2.6542	0.04002	0.012 *
	Soil:Water	2.1465	0.03237	0.026 *
	NeW Vs NeCK	2.5809	0.105	0.002 **
	AcW Vs AcCK	3.0537	0.12189	0.001 ***
PacBio	Soil	0.9707	0.01801	0.358
	Water	7.9983	0.14838	0.001 ***
	Soil:Water	0.9332	0.01731	0.386
	NeW Vs NeCK	2.5963	0.10556	0.001 ***
	AcW Vs AcCK	7.9223	0.26476	0.001 ***

Taxonomic comparison of different sequencing methods

To determine whether differences in sequencing regions affected the assignment of sequences to different taxonomic levels, we analyzed the annotation proportion of forty-eight datasets for V4, LoopSeq, and PacBio each, at phylum, class, order, family, genus, and species levels. The results showed that the classification resolution on all the taxonomy levels of different sequencing methods was significantly different ($P < 0.05$) (Fig. 2ACE; Fig. S1ACE). At the phylum level, the proportion of assigned sequences was ranked as V4 > LoopSeq > PacBio (Fig. 2A), and at the class level, the proportion of assigned sequences was ranked as PacBio > V4 > LoopSeq (Fig. S1A). However, at other levels, the PacBio data sets had the highest proportion of assigned sequences (Fig. 2B and E; Fig. S1C and E). Venn analysis showed that the numbers of shared phylum, genus, and species were 16, 201, and 24, respectively, among three different sequencing methods. Furthermore, the numbers of the unique genera and species of LoopSeq and PacBio sequencing were significantly higher than those of V4 sequencing. The numbers of shared class, order, and family were 33, 58, and 115, respectively, among the three different sequencing methods (Fig. 2B DF, Fig. S1BDF).

Rhizosphere microbial community structure of different sequencing methods at the phylum level

Analysis of phylum-level showed that the effect of waterlogging on the rhizosphere soil microbial relative abundance of the soybean on the two types of soil was different among the three sequencing methods. A total of 44 phyla were detected by V4 sequencing, among which *Proteobacteria*, *Acidobacteria*, and *Chloroflexi* were dominant, with relative abundances ranging from 31.1–39.1%, 15.0–21.9%, and 5.9–7.3%, respectively. A total of 36 phyla were detected by LoopSeq sequencing, among which *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the most pronounced, with relative abundances from 33.5–40.3%, 18.4–25.7%, and 7.6–10.8%, respectively. PacBio sequencing detected 29 phyla, among which *Proteobacteria*, *Planctomycetes*, and *Bacteroidetes* were dominant, with 30.7–35.4%, 15.9–21.1%, and 9.8–12.8%, as their relative abundances, respectively (Fig. S2). Waterlogging increased the relative abundance of *Firmicutes* and *Gemmatimonadetes* in the two types of soil, but the increase was significantly higher on the acidic than in the neutral soil for the three sequencing methods ($P < 0.05$) (Fig. S3J and K).

Rhizosphere microbial community structure of different sequencing methods at the genus level

Discrepancies among microbial community profiles represented by different sequencing methods were obvious at the genus level (Fig. 3). The first 30 genera with higher relative abundance were selected from the shared 201 genera from the three methods. Waterlogging was shown to increase the relative abundance of *Pirellula* in the two types of soil in only the PacBio sequencing method. However, the relative abundance of *Geobacter* was increased by waterlogging in all the sequencing methods.

Rhizosphere microbial community structure of different sequencing methods at the OTU level

Differential expression analysis of OTUs was also performed. In V4 sequencing, waterlogging increased 60 OTUs that were shared amongst both neutral and acidic soils ($P < 0.05$). These OTUs were classified at the genus level and were mainly composed of *Geobacter* (7 OTUs, 2.4%), *Nitrospira* (1 OTU, 0.7%), and *Anaeromyxobacter* (3 OTUs, 0.4%) (Fig. S4A). In LoopSeq sequencing, waterlogging increased 10 OTUs that were shared between neutral and acidic soils ($P < 0.05$). Classification of these OTUs at genus level showed that most of them were *Oryzihumus* (1 OTU, 0.5%), *Massilia* (1 OTU, 0.18%), and *Acidothermus* (1 OTU, 0.16%) (Fig. S4B). Waterlogging increased the 9 OTUs in PacBio sequencing that were shared between the two types of soil ($P < 0.05$). These OTUs were classified at the genus level and were mainly made up of *Flaviolibacter* (1 OTU, 0.67%), *Ramlibacter* (1 OTU, 0.64%), and *Geobacter* (4 OTUs, 0.43%) (Fig. S4C). After assigning these OTUs to the genus, we did not find the co-enriched microbial species in the three sequencing methods (Fig. S4D).

Core microbiome analyses

There were 18, 4, and 10 OTUs in V4, LoopSeq, and PacBio sequencing, respectively (Table S1). In V4 sequencing, the core OTUs only accounted for 0.69% of the total number of OTUs. *Proteobacteria* was the most abundant in the core rhizosphere microbial community, and was composed of 8 OTUs, and accounting for 5.2% of the average relative abundance. Other OTUs represented in the core rhizosphere microbiome were 5 *Acidobacteria* OTUs (1.56% relative abundance), 1 *Bacteroidetes* OTU (0.99% relative abundance), 1 *Firmicutes* OTU (0.61% relative abundance), 1 *Gemmatimonadetes* OTU (0.54% relative abundance), 1 *Nitrospirae* OTU (0.32% relative abundance) and 1 *Verrucomicrobia* OTU (0.32% relative abundance). (Fig. 5A). In LoopSeq sequencing, core OTUs only accounted for 0.061% of the total number of OTUs. This core rhizosphere microbiome consisted of 2 *Actinobacteria* OTUs (1.24% relative abundance) and 2 *Firmicutes* OTUs (1.12% relative abundance) (Fig. 5B). In PacBio sequencing, the core OTUs represented only 0.026% of the total number of OTUs. This core rhizosphere microbiome had 4 *Proteobacteria* OTUs that made up 2.09% of the mean relative abundance. Other OTUs in this core rhizosphere microbiome were 2 *Bacteroidetes* OTUs (1.18% relative abundance), 1 *Nitrospirae* OTU (0.36% relative abundance), and 1 *Planctomycetes* OTU (0.31% relative abundance) (Fig. 5C). Among them, OTU4 (*Bacillus*) and OTU4746 (*Bacillus*) were the core species shared by V4 and LoopSeq sequencing, while OTU17 (*Nitrospira*) and OTU22 (*Nitrospira*) were core species shared by V4 and PacBio sequencing.

Modular analysis of the co-occurrence network

Network modeling was applied to assess the composition and structure of microorganisms with different sequencing methods, and the OTUs with sequence numbers > 5 were screened as nodes. The entire networks of the V4, LoopSeq, and PacBio datasets were each divided into seven major modules. In the V4, modules I, II and III accounted for 35.85%, 32.76%, and 28.45% of the whole network, respectively (Fig. 6A). In LoopSeq, modules I and II accounted for 32.98% and 14.61% of the whole network, respectively (Fig. 6B) whereas in the PacBio, modules I, II, III, and IV accounted for 28.82%, 22.94%, 20.59%, and 20% of the whole network, respectively (Fig. 6C).

To evaluate the differences in OTUs on different soils and different waterlogging treatments under the three sequencing methods, we created a generalized linear model of the negative binomial distribution to analyze modules with high percentages. OTU numbers in the rhizospheres of three sequencing methods under the non-waterlogging and acidic soil were used as the controls to compare the enriched or depleted OTUs under the waterlogging and neutral soil, respectively (Table S3).

The volcano plot of V4 sequencing showed that all OTUs of module II and module III had higher relative abundances in the waterlogging than non-waterlogging, whereas all the OTUs of module I had lower relative abundances in the waterlogging treatments than that of non-waterlogging treatments. The most OTUs of modules I, II, and III had higher relative abundances in the neutral soil than acidic soil. In LoopSeq sequencing, the 4 OTUs of the module I had higher relative abundances in the waterlogging treatments than in non-waterlogging treatments, whereas the 2 OTUs of module I and all OTUs of module II had lower relative abundances in the waterlogging treatments. Most OTUs of module I and 4 OTUs of

module II had higher relative abundances in the neutral soil than acidic soil. For PacBio sequencing, all OTUs of modules II, III, and IV had higher relative abundances in the waterlogging treatments than non-waterlogging treatments. All OTUs of the module I had lower relative abundances in the waterlogging treatments than in non-waterlogging treatments. The 8 OTUs of module I had higher relative abundances in the neutral soils than acidic soils, whereas most OTUs of module I and all OTUs of modules II, III, and IV had lower relative abundances in the neutral soils than acidic soils.

Nodes with high node degree, closeness centrality, and betweenness centrality were defined as the key species of the rhizosphere network (Table S2). In general, OTU7370 (*unclassified_Sphingobacteriales*), OTU405 (*unclassified_Verrucomicrobia*), OTU2287 (*unclassified_Ellin6513*), and OTU769 (*unclassified_SJA-28*) were identified as keystone species for V4 sequencing, while OTU1516 (*Solirubrobacter*), OTU5896 (*Conexibacter*), OTU791 (*Geobacter*), and OTU3146 (*Massilia*) were key species for the LoopSeq sequencing. For the PacBio sequencing, the key species were OTU17 (*Gemmatimonas*), OTU18 (*Flavisolibacter*), OTU355 (*Aquisphaera*), and OTU10711 (*Algisphaera*).

Environmental drivers of different sequencing methods

To determine the correlation between soil properties and microbial communities, we conducted a paired comparison of environmental factors. Our results showed that V4, LoopSeq, and PacBio sequencing had significant correlations with all soil physical and chemical factors (Fig. 7A). As the physical and chemical properties of the two soils were quite different, we compared the relationship between the physical and chemical properties of the two soils and the microbial communities of the three sequencing methods, respectively. In neutral soil, V4 sequencing was significantly correlated with AP ($P < 0.05$), and extremely significantly correlated with NO_3^- ($P < 0.01$). LoopSeq sequencing was only significantly correlated with NO_3^- ($P < 0.05$). PacBio sequencing was only extremely significantly correlated with NO_3^- ($P < 0.01$) (Fig. 7B). In acidic soil, V4 sequencing was extremely significantly correlated with NH_4^+ and NO_3^- ($P < 0.01$). LoopSeq sequencing was only extremely significantly correlated with AP ($P < 0.01$). PacBio sequencing was significantly correlated with AP ($P < 0.05$) and extremely significantly correlated with NH_4^+ and NO_3^- ($P < 0.01$) (Fig. 7C).

Discussion

In this study, we used three sequencing methods to evaluate the impact of waterlogging on the structure of soybean rhizosphere microbial communities on two types of soil. Our first hypothesis has not been fully verified, as the waterlogging changed the microbial community structure and not the diversity in both types of soil irrespective of the sequencing method. Moreover, we had hypothesized that the resolution of full-length sequencing was higher than that of partial sequencing. However, this could not be fully verified, at the phylum level, the resolution of partial sequencing is higher than that of full-length sequencing, and the class level resolution of LoopSeq sequencing is lower than that of partial sequencing. Although both LoopSeq and PacBio are full-length sequencing, the resolution of PacBio sequencing was higher than that of LoopSeq sequencing except at the phylum level (Fig. 3 and Fig. S3).

Based on CPCoA analysis, the results from all the analyzed sequencing methods showed that waterlogging significantly affected the rhizosphere bacterial community structure (Fig. 1C, D and E) and this was in agreement with the arguments put forward by [51]. When the soil is waterlogged, the oxygen content of the soil sharply decreases which reduces the respiration rate and activity of soil microorganisms. This in turn leads to the expected changes in microbial community structure [52–55]. Furthermore, changes in crop root exudates induced by waterlogging also directly affected rhizosphere microbial community structure [56]. Crops suffering from waterlogging stress affect the underground carbon input [57, 58], which then affect the rhizosphere microbiome [59]. We selected acidic soil and neutral soil in this study with an expectation that soil could significantly affect the rhizosphere microbial community structure, irrespective of the sequencing methods. However, we found that soil type was not a significant factor driving changes in microbial community structure in PacBio sequencing. This could possibly be attributed to long sequencing which could lead to the reconstruction of phylogeny and thus affecting the similarities or differences of microbial communities [60].

Our results showed that full-length sequencing (except at the phylum level) had a higher classification resolution (Fig. 3; Fig. S1). This was anticipated, as full-length reads sequence has been shown to provide a higher phylogenetic classification resolution [61]. When sequencing with different variable regions, almost all the sequences of V1-V9 were annotated to species level compared with other variable regions [27, 62]. Because full-length sequencing covers most of the target genes, it has a high-resolution capacity to discriminate many phylogenetic closely related taxa [63, 64]. However, the resolution of LoopSeq was lower than PacBio, which could be due to differences in the sequencing platform. LoopSeq uses the Illumina platform for full-length sequencing. PacBio's CCS library can improve the accuracy by sequencing a single fragment for multiple rounds leading to a more accurate species classification [62]. Similar to previous studies [65], we found that the classification of microbial groups is affected by a smaller 16S amplicon. The V4 datasets suffered from this biasness, which further supported the use of longer readings for microbial ecological analysis [66].

To determine whether the high species classification resolution of full-length sequencing could help in identifying more microorganisms related to waterlogging resistance, we compared the microbial community structure on the phylum and genera levels using three sequencing methods. Our results revealed that *Verrucomicrobia* and *Planctomycetes* were abundant in V4 and PacBio, respectively. The soil under waterlogging stress produces a lot of methane [67]. *Verrucomicrobia* and *Planctomycetes* can participate in the synthesis of formaldehyde oxidation-related enzymes in the methane oxidation pathway [68, 69]. Some *Verrucomicrobia* can ferment various sugars under anaerobic conditions, and provide nutrients for plants [70]. For the different phylum in the two soil types, a kind of phototrophic *Gemmatimonadetes* bacteria was enriched in acidic soil in all three sequencing methods, which was consistent with previous studies [71].

The effect of waterlogging on the rhizosphere soil bacteria at the genus level was different across the sequence methods. For example, *Variovorax* was only detected in V4 sequencing and has been previously reported to manipulate plant ethylene levels to balance the normal root development [72], thus avoiding

the harm of waterlogging [73]. The increased relative abundance of *Pirellula*, which plays an important role in nitrogen cycling, was only found in PacBio sequencing [74, 75]. These different microbial species detected by different sequencing methods might affect our screening of waterlogging tolerance-related microorganisms. However, we still found some same trends in some microbial genera that respond to the waterlogging among the three sequencing methods (Fig. 4). The increased abundance of *Geobacter* in waterlogging stress was detected in the three sequencing methods. *Geobacter* plays an important role in plant nitrogen fixation [76, 77], and can secrete fulvic acid and participate in plant electron transfer [78–80], that may be related to electrical signals mediated by plant potassium channels [81]. The hypoxic environment under waterlogging stress results in a sharp decline in the microorganisms involved in the nitrification reaction. This inhibits the activity of the nitrifying community leading to increased nitrogen loss [82]. However, the enrichment of anaerobic bacteria (such as *Geobacter*) may fix more nitrogen, thereby allowing plants to grow healthily. However, the extent to and the mechanism through which *Geobacter* improves the adaptability of a plant to waterlogging stresses remains unknown and needs to be explored in the future.

At the OTU level, the effect of waterlogging on the two soils was also different among the three sequencing methods. The OTUs, that significantly changed in both types of soil after waterlogging belonged mainly to *Geobacter*, *Anaeromyxobacter*, and *Nitrospira* in V4, *Oryzihumus*, *Massilia* and *Acidothermus* in LoopSeq, and *Flavisolibacter*, *Ramlibacter* and *Geobacter* in PacBio. Among them, *Geobacter* was observed in both V4 and PacBio sequencing methods, which was consistent with analysis on the genus level. Previous studies have shown that *Geobacter* and *Nitrospira* are related to microbial nitrogen fixation [77, 83]. These genera are reductive microorganisms [84], which can use a wide range of carbon and/or electron donors to participate in metabolic pathways. The broad metabolic diversity of microorganisms was considered to be advantageous, particularly at times of nutrient scarcity [85]. However, long sequencing revealed more differences in OTU that have other functions. Some microorganisms identified by LoopSeq and PacBio are related to the phosphorus cycle and high soil fertility. For instance, *Massilia* may help to the turnover of root exudates, such as amino acids, sucrose, and fatty acids, and may provide phosphorus solution to plants [86, 87]. *Acidothermus* can decompose organic matter and utilize carbon sources thus enriching the soil organic matter content [88]. *Flavisolibacter* has an effect of dissolving phosphorus in the soil [89, 90]. Therefore, long sequencing may detect more microbial information related to waterlogging tolerance.

Core microorganisms with different functions are involved in the coordination and organization of plant-microbe interactions [91]. Three sequencing methods resulted in different species of core microbiome which mainly included *Nitrospira*, *Geobacter*, *Variovorax*, and *Bacillus* in V4 sequencing, *Bacillus* and *Dactylosporangium* in LoopSeq sequencing, and *Nitrospira*, *Flavisolibacter*, *Gemmatimonadetes* and *Ramlibacter* in PacBio sequencing (Fig. 5). *Nitrospira* was the core microorganism shared by V4 and PacBio sequencing, while *Bacillus* was the core microorganism shared by V4 and LoopSeq sequencing. *Nitrospira* is the most common genus affecting soil nitrogen metabolism [92–94]. *Bacillus* can utilize multiple electron donors or collectors to enrich nutrients [95] and maintain normal root growth [72]. Besides, *Geobacter*, which is related to nitrogen fixation [77, 83], and *Flavisolibacter*, which can dissolve

soil phosphorus [89, 90], were the core microorganisms for V4 and Pacbio sequencing, respectively. These core microorganisms might help plants resist waterlogging stress through different nutrients cycles or recruit other beneficial microorganisms to resist the effects of waterlogging together with plants. Nevertheless, whether the core microorganisms we discovered could establish a defense mechanism against waterlogging damage with soybeans is still unclear and requires further experimental verification.

Co-occurrence patterns are ubiquitous in nature and particularly are involved in the analysis of microbial community structure. Network co-occurrence analysis can provide an in-depth and unique perspective for understanding microbial interactions and ecosystem assembly rules, rather than simple species diversity and composition [96–98]. Network modularity may reflect collaborative relationships, competitive interactions, and niche differentiation, which leads to non-random patterns of interaction and affects the complexity of the ecological network [99]. Dividing the network into modules helps to clarify different node groups that perform different functions [100]. For example, the main modules with a high percentage in V4 (except module I) and LoopSeq sequencing are enriched with some microorganisms related to the nitrogen cycle (e.g., *Mucilaginibacter*, *Candidatus Solibacter*, *Candidatus Koribacter*, *Geobacter* and *Bacillus*) after waterlogging [101, 102]. This agrees with previous studies that the nitrogen-fixing microorganisms might be enriched in the waterlogging soil [103, 104]. Moreover, the main modules with a high percentage of LoopSeq and PacBio sequencing are enriched with some microorganisms related to the phosphorus cycle (e.g., *Massilia* and *Flavisolibacter*) after waterlogging [89, 90]. This showed that waterlogging can selectively increase or decrease part of the microbial abundance related to the nitrogen cycle. However, the functions of depleted microorganisms in the main modules of LoopSeq and Pacbio sequencing have not been reported.

Compared with acidic soil, the microorganisms related to nitrogen fixation (e.g., *Geobacter*, *Nitrospira*, *Candidatus_Koribacter*, and *Candidatus Solibacter*) in the main modules of the network are enriched in neutral soil [76, 101–106]. This might indicate that waterlogging is less harmful to neutral soils than acidic soils, at least on the level of the microbial functions. However, microorganisms related to the phosphorus cycle (e.g., *Flavisolibacter*, *Massilia*, and *Gemmatimonas*) were depleted in neutral soils [89, 90, 92]. In this study, acidic soil had lower phosphorus content than neutral soil. A previous study showed that when P availability in soil is low, the enrichment of inorganic phosphate-solubilizing bacteria could efficiently transform immobilized P into bio-available P with high phosphatase activities [107]. Moreover, the keystone species in the rhizosphere varied among V4, LoopSeq, and PacBio sequencing, which might be a key determinant of the composition of other communities in the rhizosphere of plants [108].

To determine the environmental factors affecting the microbial communities of three sequencing methods in different soils, we performed Pearson's correlation coefficients analysis using all samples from both types of soil. The results showed that all the environmental factors affected the microbial community in both types of soil in the three sequencing methods. This might have been caused by the soil heterogeneity between neutral and acid soils [109]. In neutral soil, NO_3^- was the main environmental factor that affected the microbial community in all sequencing methods. Previous studies have shown

that some period after the waterlogging, the soil nitrogen form is still dominated by NO_3^- , which could be transported into the host by microorganisms [110, 111]. For the acidic soil, NH_4^+ and NO_3^- were the major environmental factors that affected the microbial community in V4 and PacBio sequencing. TP affected the microbial community in LoopSeq and PacBio sequencing methods. These results were in line with the network module association analysis, which showed that phosphorus-related microorganisms were enriched in acidic soil. It has been previously reported that soil microbial community structure was significantly affected by soil phosphorus content [112]. From these results, the impact of environmental factors on the microbial community was different among the three sequencing methods and with the soil type.

Conclusion

In conclusion, this study compared three sequencing methods to analyze the impact of different soil and waterlogging on soybean rhizosphere microbial communities. Based on our findings, the resolution of full-length sequencing is higher than that of partial sequencing. But this was not observed at phylum level of three sequencing methods and class level of LoopSeq sequencing, and the ability of different sequencing methods to identify species of community is also different. CPCoA showed that waterlogging has significant effects on rhizosphere microbial community structure in the V4, LoopSeq, and PacBio sequencing. However, for the V4 and LoopSeq sequencing, soil type also has a significant effect on rhizosphere microbial community structure. Results from all three sequencing methods show that the waterlogging on both types of soil increases the relative abundance of *Geobacter*. However, full-length sequencing detected microorganisms related to the phosphorus cycle, such as *Flavisolibacter* and *Massilia*. Moreover, the environmental and ecological determinants of microbial community structure in different sequencing methods are different. According to our results and previous studies, full-length sequencing is the best choice for understanding the ecological functions of microorganisms as it allows for accurate classification and microbial analysis. The use of PacBio sequencing technology results in more accurate identification of the complex microbial community based on full-length bacterial 16S rRNA gene. However, the high cost limits many scientists from using it. LoopSeq sequencing is a much cheaper full-length sequencing method and can be operated with a kit.

Abbreviations

NeCK: Soybean rhizosphere soil without waterlogging in neutral soil; NeW: Soybean rhizosphere soil with waterlogging in neutral soil; AcCK: Soybean rhizosphere soil without waterlogging in acidic soil; AcW: Soybean rhizosphere soil with waterlogging in acid soil. V4: Illumina Miseq; LoopSeq: Full-length Loop Genomics sequencing technology; PacBio: Full-length the PacBio single molecule, real-time (SMRT) technology.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the raw data of the V4 16S sequence, LoopSeq sequencing, and PacBio sequencing were deposited in the NMDC under accession number NMDC10017771, NMDC10017785, and NMDC10017787, respectively.

Competing interests

The authors declare that they have no competing interests.

Author' contributions

YT, LT and NN conceived and designed the experiments. YT, LT, CL, LQ and WS performed the experiments. ZY, ZH and TM completed the library preparation and sequencing. YT, LT and NN analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

Effects of waterlogging and soil types on soybean rhizosphere soil bacterial hao1 (A) and Shannon index (B) with Illumina Miseq, LoopSeq, and PacBio full-length sequencing method (one-way ANOVA, $n=6$, $P < 0.05$). Constrained Principal-coordinate analysis (CPCoA) based on Bray-Curtis distance showing differences in rhizosphere bacterial community structure under waterlogging in neutral and acidic soil (PERMANOVA, $n=6$, $P < 0.05$) (CDE). Different letters indicate significant differences ($P < 0.05$).

Figure 2

Taxonomy profiles in different sequencing methods datasets. The proportion of annotation sequences from the V4 (n =48, blue), LoopSeq (n= 48, yellow), and PacBio (n =48, orange) datasets was determined by comparing the sequence with the SILVA database, and are represented at the phylum (A), genus (C), and species levels (E). Venn diagram showing the numbers of unique and shared phylum (B), genus (D), and species (F) between three sequencing methods. Blue denotes V4, yellow denotes LoopSeq, and orange denotes PacBio.

Figure 3

Relative abundance analysis of common genus in three sequencing methods. The most abundant 30 genera were selected from shared 201 genera of the three sequencing methods. Color pairs denote samples of three sequencing methods in neutral or acidic soil with different waterlogging times. Bubble sizes indicate the read abundance of an individual genus.

Figure 4

Rhizosphere core microorganisms of different sequencing methods. The different parts inside the double pie chart represent the bacterial phyla of the soybean core microbiome. The different parts outside the double pie chart represent the OTU (genus) of the soybean core microbiome, and each OTU (genus) is assigned to the corresponding bacterial phyla. The size of the different double pie chart portions represents the percentage of phylum/genus relative abundance in all core microbial components.

Figure 5

Network analysis reveals the symbiotic pattern between OTUs (A, B, C). The nodes are colored according to the modular type. The connections between nodes indicate strong and significant (Spearman's $r > 0.8$ or $r < -0.8$) ($P < 0.01$) correlation. The volcano map shows the amount of OTU enriched and depleted in neutral soil and after waterlogging in the modules of different sequencing methods, respectively. Violet denotes Module I, green denotes Module II, blue denotes Module III, black denotes Module IV, orange denotes Module V, red denotes Module VI, cyan denotes Module VII, grey denotes other modules.

Figure 6

Paired comparison of environmental factors and microbial community with a color gradient denoting Pearson's correlation coefficient. Spearman's correlation coefficient > 0 indicates positive correlation and < 0 indicates negative correlation. Effects of environmental factors in two types of soil (A), neutral soil (B), and acidic soil (C) on the microbial communities of the three sequencing methods. The edge width corresponds to the distance dependence of Mantel's R statistic, and Statistical significance based on

9,999 permutations represents edge color. Mantel's r size indicates the strength of the correlation. The color of the connecting line indicates the correlation between different sequencing methods and environmental factors.

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