Structural And Functional Changes In Soil Microbes By Foliar Drift Spray of Seaweed Extract As Revealed By Metagenomics

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Abstract

Kappaphycus alvarezii seaweed extract (KSWE) is known to enhance crop productivity and impart stress tolerance and our preliminary studies showed their biostimulatory effect on soil bacteria also. Close to one quarter of the foliar spray carried out on maize falls on soil either as drift or from leaf as drip. Hence it was hypothesized, it would profoundly influence soil microbes under stress. An experiment was conducted with five treatments, with or without KSWE application at critical stages of maize under soil moisture stress and compared with an irrigated control. Illumina platform was employed for analysis of V3-V4 region of 16S rRNA gene from the soil metagenome. Total of 345,552 operational taxonomic units were generated which were classified into 55 phyla, 152 classes, 240 orders, 305 families and 593 genera. Shannon's index and Shannon's equitability indicated increased soil bacterial diversity by multiple KSWE applications under duress. The abundance of *Steroidobacter*, *Balneimonas*, *Rubrobacter*, *Bacillus*, *Alicyclobacillus*, *Anaerolinea* and *Nitrospira* genera decreased (49-79%) in drought imposed at V5,10, and 15 stages of maize over the irrigated control, while it significantly improved when followed by KSWE application under drought. *Nitrosomonas*, *Nitrosovibrio*, *Rubrobacter*, *Flavobacterium* genera and several other taxa which are important for plant growth promotion and nutrient cycling were found to be enriched by KSWE application under drought. Treatments having enriched microbial abundance due to KSWE application under stress recorded higher soil enzymatic activities and cob yield, suggesting the contribution of altered soil ecology mediated by KSWE as one of the reasons for yield improvement.

Introduction

Reports on improvement in crop yields and stress alleviation following application of seaweed-based biostimulants are on the rise (Dalal et al., 2019; Garai et al., 2019; Rouphael et al., 2018; Trivedi et al., 2017). *Kappaphycus alvarezii* seaweed extract (KSWE) is one such tropical seaweed-based biostimulant, the application of which enhances economic yield of many crops such as rice (Pramanick et al. 2014; Sharma et al. 2017; Layek et al. 2018), wheat (Patel et al. 2018), soybean (Rathore et al. 2009), sugarcane (Karthikeyan and Shanmugam 2017; Singh et al. 2018), green-gram (Raverkar et al. 2016), black-gram (Pramanick et al. 2016), potato (Pramanick et al. 2017), and tomato (Zodape et al. 2011). Around 60% of food for the global human population is catered by wheat, rice and maize (Tilman et al. 2002) and to target their higher productivity with low carbon footprint has significance in terms of reducing Global warming potential (Ghosh et al. 2015; Sharma et al. 2017; Singh et al. 2018). In maize, enhancement in grain yield by foliar application of KSWE and the possible underlying physiological, molecular and agronomic basis of improvement have been reported earlier by us (Layek et al. 2015; Mondal et al. 2015; Singh et al. 2016; Kumar et al. 2019). It was also reported that KSWE application partly alleviated soil moisture stress in maize by modulating antioxidant enzymes and differential expression of certain genes (Trivedi et al. 2018a; Trivedi et al. 2018b). However, the effect of foliar spray of KSWE, or rather any seaweed based biostimulant on soil microflora has never been reported. This has been probably neglected because the drift spray and drip from leaves falling onto the soil is seemingly miniscule. However, a careful quantification revealed that on average a quarter of biostimulants applied as foliar spray reaches the soil over the crop cycle (Table 1) and such a significant amount of it may have a direct effect on soil microflora and related soil biochemical processes, which formed the hypothesis of the present work. The study assumes, maintenance of soil fertility and structure is controlled by interactions of a highly assorted and complex web of soil microflora and fauna (Davet 2004). Enzymes released/produced by soil microbes are responsible for organic matter decomposition and nutrient cycling (Quan and Liang 2017). It was also hypothesized that the influence of seaweed based biostimulants on soil bacteria would be manifested more under soil moisture stressed condition as drought profoundly influences the dynamics of the microbial communities of soil (Quan and Liang 2017). Whether or not the seaweed biostimulant influences the soil bacteria positively under abiotic stress condition also formed the basis of present work. Accordingly, the study was conducted with the objective of assessing whether management practice involving the foliar application of KSWE influences the soil culturable and unculturable bacterial community composition beneficially. This was carried out through the amplicon-based sequencing of 16S rRNA genes through nextgen Illumina sequencing. In addition, its effect on the yield of maize crop under normal as well as drought stress conditions was also evaluated.

Table 1
Spray and drift volumes of KSWE at critical growth stages of maize along with leaf area at the time of spraying (numbers presented are mean of three replicates).

Spraying stage	Leaf area (cm^2 $plant^{-1}$)	Total volume of KSWE sprayed (ml $plant^{-1}$)	Volume retained on plant leaves (ml $plant^{-1}$)	Volume drifted on soil (ml $plant^{-1}$)	% of KSWE drifted on soil
V5	454 ± 57	9.3 ± 1.2	5.06 ± 0.49	4.28 ± 0.75	45.6 ± 3.12
V10	2478 ± 275	19.0 ± 1.7	14.69 ± 1.72	4.31 ± 0.61	22.8 ± 3.36
V15	3259 ± 594	30.0 ± 0.0	25.10 ± 0.13	4.90 ± 0.13	16.3 ± 0.42

Materials And Methods

2.1. Preparation of KSWE

The extract was prepared in bulk quantity as per the procedure described in Trivedi et al. (2017), stored at 4°C and used as and when required. Details of the composition of KSWE have been described earlier in Singh et al. (2016) and the same batch of the seaweed extract was also used in the present experiment. The total soluble solids (TSS) of the KSWE was in the range of 3–4 %.

2.2. Ethics Statement

There was no specific permission required for sampling in the experimental area of this study. The location is not protected or privately owned in any way, and it is confirmed that our experiment did not involve endangered or protected species.

2.3. Experimental site and design

Two preliminary experiments followed by metagenomic study were conducted in pots at the net house facility (21°44'57.6"N latitude, 72°08'39.3"E longitudes) of CSMCRI, Bhavnagar district, Gujarat, India, during the *rabi* season (November 2014 – March 2015).

In a preliminary experiment 1, Plant Growth Promoting Rhizobacteria (PGPRs), namely, *Bacillus subtilis*, *Bacillus licheniformis* representing *Bacillus* family and *Pseudomonas fluorescens* representing *Pseudomonas* family, were individually tested for bacterial growth enhancement upon KSWE application. For this, two concentrations of KSWE (5 and 10%) were added to a bacterial culture containing initial count of 10^5-10^6 colony forming units (CFU) ml^{-1}. Nutrient broth medium without added KSWE was treated as control. Final colony counts were recorded through standard plate count technique after 4 h incubation (at 37°C, 120 RPM) of respective treatments along with its control (in triplicate).

In another preliminary experiment (Experiment 2), total bacterial counts in soil were recorded (in triplicate) employing plate count method on 12th day after single KSWE application to soil at 5 and 10% concentrations under normal as well as drought conditions in pots grown with maize. The volume of KSWE applied was 1.5 ml kg^{-1} of soil. The drought was induced by withholding irrigation for the entire duration up to sampling time. The result of these two preliminary experiments formed the basis for further more elaborate metagenomic analysis of soil bacteria.

The metagenomic experiment consisted of 5 treatments *viz*., T1 – water spray once at V5 stage of maize (variety: Sugar 75, Syngenta) along with transient drought stress (sample code: K1, K2, K3), T2 – KSWE foliar application once at V5 along with transient drought stress (sample code: K4, K5, K6), T3 – three water sprays at V5, V10 and V15 stage with transient drought stress at all the three stages (sample code: K7, K8, K9), T4 – three KSWE foliar applications at V5, V10 and V15 stage with transient drought stress at all the three stages (sample code: K10, K11, K12), T5 – control plants neither with spray of water/KSWE nor with transient drought (sample code: K13, K14, K15). In the metagenomic study, KSWE was applied at 10% concentration which was found to be the optimum dose eliciting a favorable response in maize (Layek et al. 2015, Trivedi et al. 2017). Water spray contained the same amount of preservatives in appropriate concentrations which were present in KSWE. Transient drought condition was generated by withholding watering for 8 days till visible wilting symptoms were observed at some of the important growth stages viz., V5, V10, and V15 identified as critical stages in maize for application of KSWE in our earlier experiments with V5 being the most critical (Trivedi et al. 2017). Except for the drought period, plants were watered at the rate of 2 liters per pot every alternate day in all the experiments. The treatments were distributed in a completely randomized design (CRD) with three replications.

In addition to this, one normally irrigated treatment was kept where KSWE was sprayed daily in between V5 to grain filling stage just to know the detrimental effect of seaweed extract on soil microbes, if at all, when sprayed daily (T6; sample code: K16). However, this treatment was in single replication and not included in any statistical comparison.

All the pots were filled with 32 kg of soil to which chemical fertilizers at the recommended rate of 120:60:40 kg ha^{-1} of $N/P_2O_5/K_2O$ were applied uniformly to all the treatments through urea, single super phosphate, and sulfate of potash, respectively. The initial soil of the experiments was sandy loam in texture, having pH 7.80 and electric conductivity of 0.20 dS m^{-1}. Available N, P, and K were 103, 14 and 161 kg ha^{-1}, respectively. Organic carbon in soil was 0.51%. Four seeds were sown in each pot, which after successful germination was thinned to single plant per pot. The meteorological data of the experimental site is given in the Appendix S1.

2.4. Soil sampling

Core soil samples from around 30 cm depth were taken on 12th day after treatment in preliminary experiment 2 and at the time of plant harvesting for metagenomic and biochemical studies. All samples were collected in sterile 50ml flacon tubes and transported immediately to the laboratory in ice. Later they were sieved (< 2 mm) and stored at 4°C until processing. The metagenomic study was carried out from all the treatments, while soil enzyme analysis was carried out only in the two most significant treatments found in the metagenomic study (*viz*. T3 and T4) from the samples collected at harvest.

Electric conductivity (EC) and pH of the initial soil samples were determined in 1:2.5 slurry of soil: water. Organic carbon was determined by Walkley and Black procedure as described in Nelson and Sommers (1982). Available N and available P were determined according to the procedure described by Maynard et al. (2006) and Olsen's method (Olsen and Sommers 1982), respectively. K was extracted by neutral normal ammonium acetate (Hanway and Heidel 1952) and determined by using a flame photometer. Ca and Mg were determined by EDTA titrimetric method as described by Estefan et al. (2013). S was extracted with 0.15% $CaCl_2$ (Williams and Steinbergs 1959) and estimated by turbidimetric method as described by Estefan et al. (2013).

2.5. Drift volume measurements

Drift volume of KSWE solution was measured by placing a polythene cover on the top of soil in the pot during foliar application. A fixed volume of KSWE was sprayed and polythene cover was weighted before and immediately after the spraying. 1g of KSWE equal to 1 ml was used as the basis for calculating the volume. Leaf area was measured using a software called Digimizer (version 5.4.4) (Rangani et al. 2016).

2.6. Crop yield

Fresh cobs were harvested from each plant and were sundried. Dry corn weight with seeds was recorded as crop yield and expressed in g $plant^{-1}$.

2.7. DNA extraction

Bacterial genomic DNA was extracted from soil samples (0.5 g) using the PowerSoil® DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) as per the manufacturer's protocol. The initial homogenization was carried out in a FastPrep-24 homogenizer for 30 s at an amplitude of 5.5 m s^{-1}. The genomic DNA was tested for its quality and quantity using an Epoch microplate reader and then stored at -20°C.

2.8. Amplicon library construction

PCR amplification of extracted DNA for all the treatments in triplicate was carried out to amplify V3-V4 conserved regions of 16S rRNA gene sequences using the 16S rRNA gene primers (forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The PCR library was prepared using 2× KAPA HiFi HotStart Ready-mix PCR Kit (KAPA Biosystems®, U.S.A.) and Nextera® XT DNA library preparation kit (Illumina®, San Diego, California, U.S.A) to add Illumina sequencing adapters and dual index barcodes. First amplicon PCR was done followed by index PCR. For amplicon PCR, each 25 µl of PCR reaction contained 5 ng μl^{-1} (2.5 µl) of genomic DNA template, 12.5 µl of 2× KAPA HiFi HotStart Ready Mix, and 5 µl of each primer (1 µM). The 96 well plate was sealed and PCR reactions were performed in a thermal cycler with an initial denaturation step at 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and ended with an extension step at 72°C for 5 min. One microlitre of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size of amplified DNA.

For index PCR, each 50 µl of PCR reaction contained 5 µl of DNA, 5 µl of each Nextera XT Index Primers, 25 µl of 2x KAPA HiFi HotStart ReadyMix and 10 µl of PCR grade water. PCR reactions were performed in a thermal cycler with an initial denaturation step at 95°C for 3 min followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and ended with an extension step at 72°C for 5 min. One microlitre of a 1:50 dilution of the final library was run on a Bioanalyzer DNA 1000 chip to verify the size.

2.9. Illumina sequencing

All samples were subjected to paired-end (250 bp) sequencing (V3-V4 region) using Illumina MiSeq sequencer and analyzed into the MiSeq Reporter on-system software at SciGenom Labs Private Limited, Cochin, Kerala, India. Base quality checking of each cycle for all the samples was carried out and low-quality bases were removed so as to ensure that the majority of the reads with a high-quality score above 30 ($Q > 30$) were used for further downstream processing. It was found that nearly 75% of the total reads have a Phred score greater than 30 ($> Q30$, error-probability ≥ 0.001). Base composition and GC content distribution are also summarized in figures but not included in the paper.

All the sample reads were converted to FASTA files and were pooled together. The FASTA were pre-processed using the bioinformatics analysis pipeline. Chimeric sequences were removed using the program UCHIME and all non-chimeric sequences were taken for picking OTUs using the program UCLUST with a threshold of 97% of similarity (Lozupone et al. 2013; D'Argenio et al. 2014).

The Quantitative Insights Into Microbial Ecology (QIIME) program was used for the entire downstream analysis (Caporaso et al. 2010). The representative sequence was identified for each OTU and aligned against the Greengenes core set of sequences using PyNAST program (DeSantis et al. 2006a; DeSantis et al. 2006b). Further alignment was done of these representative sequences against reference chimeric data sets. The read based taxonomy classification was performed using the RDP classifier against Greengenes OTUs database.

Further, heat maps were generated using the QIIME program. The phylum, class, order, family, genus and species distribution for each sample, based on OTUs, were shown as heat maps in the Appendix S2. The taxa other than the top 10 were categorized as "Others" and the sequences that did not have any alignment against the taxonomic database were categorized as "Unknown". Rare and abundant taxa from the samples were also identified. Rare taxonomy was defined as having frequency $< 0.01\%$ and abundant taxonomy as OTUs having $> 1\%$ frequency (Galand et al. 2009; Aravindraja et al. 2013).

In order to assess the microbial diversity and distribution within and between the treatments, both α – diversity, and β – diversity were measured.

2.10. Alpha diversity and rarefaction curves

Microbial diversity within the samples was measured using Chao1, Shannon and observed species metrics. The Chao1 metric estimates the species richness while Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric is the count of unique OTUs identified in the sample. The rarefaction curves for each of the metric have been provided in Appendix S3. The metric calculation was performed using QIIME software.

2.11. Beta diversity between samples

Explicit comparisons of bacterial communities between the samples were also performed. The distance matrix was generated using a weighted and unweighted UniFrac approach. Sequence abundances were taken into account in weighted UniFrac for comparing microbial diversity. A jackknife test was performed to construct a consensus UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) tree for all samples in this set. The resulting consensus was taken for UPGMA trees built using a weighted UniFrac distance matrix.

2.12. Accession numbers

Sequencing data of all the 16 samples were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP180107) under the study SRP180107.

2.13. Soil enzyme estimation

Aryl sulphatase, acid-, alkaline- phosphomonoesterase and glucosidase activities in the field moist soil were assayed according to Tabatabai (1982) using 4-nitrophenol as standard. FDA activity was measured according to Schnürer and Rosswall (1982).

2.14. Statistical analysis

All statistical analyse were carried out using MSTAT C software. In the preliminary experiment 1, two-factor Completely Randomized Design was employed for ANOVA, while in the preliminary experiment 2 and metagenomic study one way ANOVA was used. The results were expressed in mean ± standard deviation of three independent replicates. Post hoc comparison of means was carried out using Tukey's honestly significant differences, Student-Neuman-Keul's test and Least significant different test at $p < 0.05$. The principal component analysis (PCA) was conducted using Minitab statistical software to assess the variation among treatments at different taxonomic levels. A Venn diagram was prepared using Ugent tool available on http://bioinformatics.psb.ugent.be/webtools/Venn/ to observe the identified number of unique and shared species present among all the five treatments.

Results

3.1. Bacterial colony counts

In the preliminary experiment 1, It was found that the counts of *Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas fluorescens* increased significantly with concomitant increase in the KSWE concentration (5 and 10%) and both the doses recorded higher counts over control (Fig. 1a). Subsequently in preliminary experiment 2, drought significantly reduced the total CFUs in soil while KSWE brought out a significant increase in total bacterial count under both the soil moisture regimes at 10% level (Fig. 1b). Interestingly, the total bacterial CFUs in soil under drought conditions with 10% KSWE applied was similar to that under normally irrigated soil (without KSWE). These results were a prelude to the bacterial metagenomic study for in-depth information on the diversity and ecology of soil due to the influence of KSWE.

3.2. Generation of Illumina reads

In the metagenomic study, targeting the hyper-variable V3-V4 region of 16S rRNA gene amplicons, a total of 4,314,385 OTUs were identified from 6,624,123 reads. 3,968,833 singleton OTUs (OTUs having single read) were identified and removed. After singleton removal, 345,552 distinct OTUs were found which were used for further downstream analysis. These OTUs were classified into 55 phyla, 152 classes, 240 orders, 305 families and 593 genera at a similarity threshold of 0.97.

3.3. Alpha diversity with rarefaction curves of Chao1, Shannon and Observed species

Rarefaction curves depicting alpha diversity (Appendix S3) reached near the plateau, indicating that the sampling depth and sequencing coverage were good. In addition, Shannon's index (H) and Shannon's equitability or evenness (E_H) were calculated at phylum level (Table 2) to show the relative bacterial diversity. Shannon's index revealed that there was maximum bacterial diversity when the soil was subjected to stress with concomitant KSWE application three times (T4) during the growth cycle. This was followed by that in normally irrigated soil (T5), while it was lesser in soil that was subjected to stress thrice but not treated with KSWE (T3). Further, the bacterial communities in soil were found to be more evenly distributed in T4 ($E_H = 0.5787$) and T5 ($E_H = 0.5624$) as compared to those in other treatments.

Table 2 Relative bacterial diversity as indicated by Shannon's diversity index and Shannon equitability at phylum level along with rare and abundant species and cob yield of maize plant in normal as well as drought conditions for all the treatments.

Treatments	Shannon's diversity index (H)	Shannon's equitability or evenness (E_H)	Rare species	Abundant species	Crop yield
			Total reads		(g plant^{-1})
T1- V5 water spray	1.927	0.5124	341^{b}	107234^{cd}	89.16^{c}
T2- V5 KSWE	1.929	0.516	471^{b}	119167^{c}	97.30^{b}
T3- V5,10,15 water spray	2.029	0.527	357^{b}	97812^{d}	36.33^{e}
T4- V5,10,15 KSWE	2.240	0.5787	818^{a}	224435^{b}	75.66^{d}
T5- Normal Irrigation	2.233	0.5624	911^{a}	246370^{a}	87.03^{c}
T6- Daily spray till grain filling stage	–	–	–	–	104.33^{a}

Values are mean of 3 replicates. Values followed by different alphabets in the columns are significantly different at $P < 0.05$ using Tukey's HSD.

3.4. Beta diversity with a phylogenetic tree

The bacterial community similarity, as revealed by the weighted UniFrac phylogenetic tree, is depicted in Appendix S4. It revealed that the bacterial community in the T5 (K13, K14, and K15) and T4 (K10, K11, K12) treatments were different from the T1 and T3 (K1, K2, K3, K7, K8), except K9 which clustered with the former group. The samples in these clusters (T1 and T3 vs T4 and T5) were also not close and grouped loosely indicating the difference in bacterial community composition in soil samples due to treatment effect.

3.5. Pairwise multiple comparisons of total OTU means

Significant changes ($P \leq 0.001$) in abundance of the 16S bacterial rRNA gene expressed as the number of total OTUs were detected among different treatments (T1-T5) using ANOVA and Tukey's test (Table 3). The least number of OTUs was observed in soil collected from the treatment receiving moisture stress three times during the life cycle of the crop (T3, 100157). Variation in bacterial abundance due to KSWE was evident by a significant increase in the number of OTUs in soil samples of treatment that was subjected to stress three times and also with KSWE applied (T4, 232029) over the corresponding treatment (T3) where KSWE was not applied ($P \leq 0.001$) (Table 3). Moisture stress at one or more stages significantly reduced the OTU abundance in the soil as compared to that of irrigated soil (T5, 254041), except that in case of treatment receiving KSWE three times in which case it was at par.

Table 3 Pairwise Multiple Comparison of total OTUs means (Tukey's Honestly Significant Test).

Comparison	Diff of Means	P*
T5 vs. T3	153884	<0.001
T5 vs. T1	144704	<0.001
T5 vs. T2	132290	<0.001
T5 vs. T4	22012	0.412
T4 vs. T3	131872	<0.001
T4 vs. T1	122692	<0.001
T4 vs. T2	110278	<0.001
T2 vs. T3	21594	0.43
T2 vs. T1	12414	0.837
T1 vs. T3	9180	0.936

Treatment means of total OTUs: T1: 109337; T2: 121751; T3:100157; T4: 232029; T5: 254041

*P indicates probability value. Comparisons having $P < 0.05$ were significantly different from each other.

3.6. Impact of drought and KSWE on specific bacterial groups

3.6.1. Distribution of bacterial community at phylum level

Upon taxonomic classification of all the 345,552 distinct OTUs, 9.8% of OTUs (33863 OTUs) were grouped under unclassified bacteria and the rest 90.2% were grouped into different phyla. The top 12 phyla falling within the classified OTUs in different soil samples (T1-T5) are given in Fig. 2. The 12 dominating phyla were *Chloroflexi*, followed by *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes*, TM7, *Nitrospirae*, *Verrucomicrobia* and others (which was a sum of all the rest) having individual abundance less than 0.5%.

There was a striking diversity shift with respect to the relative abundance of phyla *Proteobacteria*, *Chloroflexi* and *Firmicutes* among the treatments T3, T4 and T5 (Fig. 3). Whereas the percentage of OTUs belonging to the *Proteobacteria* decreased from 26.06% in normal irrigated treatment (T5) to 17.23% due to stress applied three times (T3), application of KSWE increased its corresponding proportion to 24.87 in the stress treatment (T4). Similarly, the proportion of the abundance (OTUs) of microbes in *Firmicutes* phylum was reduced to 9.99% when subjected to moisture stress three times compared to normally irrigated condition, but upon KSWE application three times under duress, its proportion increased to 19.42%, which was even more than that under normal irrigated treatment (15.88%). It was found that KSWE under moisture stress could bring down the relative proportion of *Chloroflexi* to an identical level (14.77%) as that under normal irrigated condition (15.9%) from the elevated level of 38.72% due to stress (T3).

The comparison of the relative abundance means of the top 12 enriched bacterial phylum categories ($P \leq 0.01$) associated with soil samples collected from T1-T5 is shown in Fig. 4. The soil samples collected from the treatments subjected to stress once or thrice and not sprayed with KSWE (T1, T3) and those subjected to stress and KSWE spray only at V5 stage (T2) had significantly decreased populations of phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia*, over the treatment that received stress and KSWE thrice (T4). KSWE applied thrice under stress (T4) showed an increase in the population of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* in soil samples by 107%, 179%, 162%, 409%, and 408% respectively, over its respective control (T3) (Fig. 4). Notably, the relative abundance of all these soil bacterial phyla in the T4 was found to be statistically similar to that under untreated normal irrigated control soil (T5). The populations of *Acidobacteria*, *TM7*, and *Nitrospirae* were not affected by any of the treatments. The abundance of OTUs belonging to *Chloroflexi* was not significantly affected by any of the treatments, however, its relative proportion vis-à-vis other phyla within a given treatment varied considerably with its proportion increasing under moisture stress. Compared to the control (T1), mere application of KSWE once in early-stage under stress at the V5 stage (T2) of the crop had no noticeable change in the abundance of any of these top 12 phyla in the soil at harvest (Fig. 4 and Table 3).

The PCA was performed to assess the variation considering 55 known and 1 unknown phylum categories. Based on the factor loadings of these phyla, the four components of PCA explained the total variations as shown in Appendix S5.

The first (PC1) and second (PC2) principal components contributed 73.9% and 10.8% of the total variation, respectively. The members of the dominant phyla including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* had high loading on PC1 indicating that these vary together in the same direction. In

contrast, *Chloroflexi* had substantial negative loading on PC1.

A biplot of the component scores has been produced indicating the second component plotted against the first component in Appendix S6. Looking at the treatment out by itself to the right, it may be inferred that the KSWE applied thrice under stress (T4) and the normally irrigated control (T5) had very high values for the first component and it is expected that these treatments would have high values for the relative abundance of the bacterial community with which they are strongly correlated, i.e., they move in a similar direction. In agreement, both these treatments had higher values for most of the enriched phylum categories. Both the water sprayed controls as well as the KSWE sprayed once (T1, T2, and T3 respectively) were located extremely left on the spectrum and thus had lower values for the relative abundance of the respective phyla.

3.6.2. Distribution of bacterial community at class, order and family level

Biplots of the distribution of bacterial communities (top ten) at the higher taxonomic levels of class, order and family also exhibited the same pattern wherein the treatments T1, T2 and T3 were distantly located compared to T4 and T5 on the first component (Appendix S6).

With respect to the top 10 enriched classes, KSWE treatment and stress applied thrice (T4) significantly improved the relative abundance of the *Alpha-*, *Beta-*, *Gamma-* and *Delta-proteobacteria* along with *Actinobacteria* and *Bacilli*, when compared to their respective water sprayed stress control (T3). Moreover, the abundance levels of these classes were brought at par to the normally irrigated treatment (T5). Among the top 10 enriched orders, relative abundance of the *Actinomycetales*, *Bacillales*, *Cytophagales*, *Myxococcales*, *Rhizobiales*, and *Xanthomonadales* followed the same trend, while, within the top 10 dominant families, a similar trend was found in *Anaerolinaceae*, *Bacillaceae*, *Cytophagaceae*, *Pseudonocardiaceae* and *Planococcaceae* (Table 4).

Table 4

Relative abundance (total OTUs) of top 10 enriched classes, orders and families as affected by all five treatments. Values represented are mean of 3 replicate
significantly different at P < 0.05 using Tukey's HSD test.

Top 10 enriched classes								
Treatments	-proteobacteria				Actinobacteria	Thermomicrobia	Clostridia	
	Alpha-	Beta-	Gamma-	Delta-				
V5 water	2663 b	620 b	1071 b	2011 b	5324 bc	2884 a	891 b	
V5 KSWE	2733 b	665 b	1302 b	2097 b	5087 bc	3520 a	1174 b	
V5,10,15 water	2874 b	816 b	1390 b	2245 b	4120 c	2747 a	1139 b	
V5,10,15 KSWE	4582 a	2054 a	2787 a	5653 a	7278 ab	536 a	2995 a	
Normal irrigation	5316 a	2359 a	3063 a	6573 a	8503 a	703 a	2502 a	
Top 10 enriched orders								
Treatments	Actinomycetales	Bacillales	Clostridiales	Cytophagales	GCA004	Myxococcales	Rhizobiales	
V5 water	5266 bc	2348 b	640 b	264 b	1600 b	1686 b	916 b	
V5 KSWE	5032 bc	2862 b	861 b	328 b	1575 b	1732 b	941 b	
V5,10,15 water	4075 c	3111 b	887 b	615 b	1439 b	1799 b	992 b	
V5,10,15 KSWE	7178 ab	8826 a	2670 a	1929 a	2138 ab	4395 a	1994 a	
Normal irrigation	8398 a	8222 a	2149 a	2133 a	2553 a	5159 a	2122 a	
Top 10 enriched families								
Treatments	A4b	Anaerolinaceae	Bacillaceae	Clostridiaceae	Cytophagaceae	Micromonosporaceae	Pseudonocardiaceae	
V5 water	4199 ab	30 b	936 b	135 b	212 b	1256 b	504 bc	
V5 KSWE	5888 a	19 b	1251 b	158 b	264 b	1156 b	529 bc	
V5,10,15 water	3660 ab	182 b	1442 b	209 b	537 b	951 b	366 c	
V5,10,15 KSWE	1980 b	904 a	3907 a	928 a	1718 a	1586 ab	620 ab	
Normal irrigation	2276 ab	873 a	3622 a	685 ab	1916 a	2214 a	761 a	

3.6.3. Distribution of bacterial communities at genus level

Relative distribution of the bacterial communities at the level of genus and species was also assessed. Top 10 enriched genus categories with their corresponding phylum have been shown in Fig. 5.

Among the top 10 genera, three genera (*Clostridium*, *Bacillus*, and *Alicyclobacillus*) belonged to the phylum *Firmicutes*. The other dominant genera were *Steroidobacter* and *Balneimonas* representing *Proteobacteria*. *Actinomadura* and *Rubrobacter* represented *Actinobacteria* while *Anaerolinea* and *Nitrospira* represented *Chloroflexi* and *Nitrospirae* phyla, respectively. The average relative abundance of the genera *Bacillus*, *Alicyclobacillus*, *Steroidobacter*, *Balneimonas*, *Rubrobacter*, *Anaerolinea*, and *Nitrospira* were significantly lower in treatment receiving moisture stress three times (T3) compared to normal irrigated T5 treatment by 59, 71, 57, 49, 63, 79 and 58%, respectively. KSWE treatment applied three times to these stress subjected treatments significantly improved the relative abundance of all these genera by 171, 196, 133, 113, 232, 416 and 126%, respectively over their corresponding control treatment (T3). Further, the normally irrigated treatment (T5) was at par with the treatment receiving stress three times along with KSWE (T4) with respect to a relative abundance of all the aforesaid genera. The relative abundance of other important soil bacterial genera having known or potential role for nitrogen fixation (*Anaeromyxobacter*, and *Methanobacterium*) and P solubilization (*Flavobacterium*) were also assessed. It was found that the KSWE application increased the relative abundance of all of these bacterial genera to that of the normally irrigated treatment. In all the aforesaid genera, the relative abundance in T4 was significantly higher compared to T3 (Appendix S7).

Principal component analysis at genus level of the top 10 and some others involved in N and P cycling also revealed that T1, T2 and T3 have similar associated abundance pattern clustered together towards the left side, while T4 and T5 clustered towards the right of the PC1, which explained 91.3% of the total variation (Appendix S5). The genera which helped distinguish the normally irrigated treatment (T5) from the treatment where KSWE and stress were applied thrice (T4) could be gauged by the influence scores in the PC2, although this component explained 6.1% of the total variation (Appendix S5).

3.6.4. Distribution of bacterial communities at species level

A Venn diagram (Fig. 6) was also prepared to observe the identified number of unique and shared species present among all the five treatments. The total number of different species identified in T1, T2, T3, T4, and T5 was 233, 260, 250, 268 and 248, respectively. The number of species unshared in these treatments were 10, 14, 9, 14 and 17, respectively. The number of species shared between all five treatments was 135. Interestingly, the number of species shared between T4 and T5 was 30, which was the highest among any other paired comparisons.

3.6.5. Abundant and rare species diversity

Both rare (frequency of species with < 0.01% of the total population) and abundant species contributed to the overall bacterial population in all the 5 treatments. Total reads for rare species and abundant species were in the range of 300 to 1000 and 97 thousand to 250,000, respectively in the treatments. Significantly higher rare and abundant species were found in T4 and T5. These treatments were at par for rare species while species abundance was more in T5 (Table 2). T1, T2, and T3 also followed a similar trend as in phylum and genus. They were significantly lower than T4 and T5 and at par with each other except T3 in the case of abundant species which was significantly lower than T2.

3.6.6. Functionally important species

Fourteen unique species found in the treatment T4 (V5,10,15 KSWE) and the top 25 most abundant species that significantly varied with the treatments were classified for their functional roles (Tables 5 and 6). The unique species found in T4 treatment are specifically known for their involvement in the processes like nitrification, denitrification, mineralization of organic compounds and production of enzymatic and non-enzymatic anti-oxidants. T4 showed significantly higher number of OTUs compared to T3 in all the 25 most abundant species shown in Table 6. Most of them were also at par with T5, a normally irrigated treatment. They were also involved in processes such as bioremediation of heavy metals, pollutants, production of antibiotic, antifungal and nematicidal compounds.

Table 5 Summary of unique bacteria identified to the species level from a 16S rRNA gene-based metagenomic study of a soil collected from V5,10,15 KSWE treatment (T4).

Phylum	Class	Order	Family	Genus	Species	OTU#	%#	
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas balearica*[a]	2	0.0029	-
	Gammaproteobacteria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter johnsonii*[b]	2	0.0034	-
	Gammaproteobacteria	*Vibrionales*	*Vibrionaceae*	*Vibrio*	*Vibrio rumoiensis*[c]	1	0.0014	-
	Gammaproteobacteria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter schindleri*[d]	2.5	0.0041	-
	Alphaproteobacteria	*Rhizobiales*	*Methylocystaceae*	*Pleomorphomonas*	*Pleomorphomonas oryzae*[e]	1	0.0014	-
	Alphaproteobacteria	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	*Paracoccus zeaxanthinifaciens*[f]	1	0.0015	-
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Pseudoclavibacter*	*Pseudoclavibacter bifida*[g]	1	0.0017	-
	Actinobacteria	*Pseudonocardiales*	*Pseudonocardiaceae*	*Actinokineospora*	*Actinokineospora diospyrosa*[h]	1	0.0014	-
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Lentibacillus*	*Lentibacillus salis*[i]	1	0.0016	-
	Bacilli	*Bacillales*	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus macerans*[j]	7	0.0097	-
Euryarchaeota	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	*Natrialba*	*Natrialba aegyptia*[k]	1	0.0017	-
	Halobacteria	*Natrialbales*	*Natrialbaceae*	*Halovivax*	*Halovivax ruber*[l]	1	0.0014	-
Bacteroidetes	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	*Marinoscillum*	*Marinoscillum furvescens*	1.5	0.0022	I
Cyanobacteria	*Cyanophyceae*	*Pleurocapsales*	*Dermocarpellaceae*	*Stanieria*	*Stanieria cyanosphaera*[m]	1	0.0015	-

#values are mean of three replicates. Alphabets written in superscripts are for showing the possible roles of species. +, Positive; -, Negative; NA, No data available

[a] Denitrifying bacterium (Ruan et al., 2020)

[b] Increase phosphate flux by synthesizing and releasing phosphates and polyphosphates (Boswell et al., 2001)

[c] Psychrophilic (cold loving) having extraordinary high catalase activity (Yumoto et al., 1999)

[d] Potentially pathogenic (Dortet et al., 2006)

[e] Nitrogen-fixing capacity (Xie and Yokota, 2005)

[f] Zeaxanthin (a common carotenoid)-producing bacterium (Berry et al., 2003)

[g] human pathogen, able to cause pulmonary disease (Oyaert et al., 2013)

[h] Contribute in recycling organic matter, Positive for having acid and alkaline phosphatase activity, an important soil enzymes for phosphate availability (Tamura et al. 1995)

[i] Catalase producing, Moderately halophilic bacterium (Lee et al., 2008)

[j] Plant growth promoting rhizobacteria (Figueiredo et al., 2008)

[k] Extremely halophilic archaea, showed desiccation and gamma radiation resistance property (Shirsalimian et al., 2017), ability to produce an extracellular polymer predominantly composed of glutamic acid (Hezayen et al., 2001)

[l] Extremely halophilic Gram-negative archaeon, catalase and oxidase positive (Castillo et al., 2007)

[m] Has photosynthetic and nitrogen fixing capacity

Table 6 Top 25 most abundant species that significantly varied across all the samples.

Phylum	Class	Order	Family	Genus	Species name	T1 (V5 water)	T2 (V5 KSWE)
Actinobacteria	*Actinobacteria*	*Streptosporangiales*	*Thermomonosporaceae*	*Actinomadura*	*Actinomadura vinacea*[1]	129^{bc}	136^{bc}
	Actinobacteria	*Actinomycetales*	*Micromonosporaceae*	*Virgisporangium*	*Virgisporangium ochraceum*[2]	57^{bc}	55^{bc}
	Actinobacteria	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	*Rhodococcus ruber*[3]	17^{bc}	15^{c}
	Actinobacteria	*Streptosporangiales*	*Streptosporangiaceae*	*Microbispora*	*Microbispora rosea*[4]	8^{b}	4^{b}
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus foraminis*[5]	85^{b}	127^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus selenatarsenatis*[6]	16^{b}	25^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus badius*[7]	26^{d}	33^{cd}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus humi*[8]	22^{c}	44^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Oceanobacillus*	*Oceanobacillus chironomi*[9]	15^{b}	12^{b}
	Bacilli	*Bacillales*	*Planococcaceae*	*Sporosarcina*	*Sporosarcina ginsengi*[10]	9^{b}	12^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus endophyticus*[11]	12^{b}	18^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Gracilibacillus*	*Gracilibacillus dipsosauri*[12]	6^{c}	15^{bc}
	Clostridia	*Clostridiales*	*Peptostreptococcaceae*	*Clostridioides*	*Clostridium difficile*[13]	10^{c}	8^{c}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus firmus*[14]	8^{b}	11^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Bacillus*	*Bacillus flexus*[15]	8^{b}	10^{b}
	Bacilli	*Bacillales*	*Bacillaceae*	*Lysinibacillus*	*Lysinibacillus massiliensis*[16]	3^{b}	3^{b}
Proteobacteria	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosovibrio*	*Nitrosovibrio tenuis*[17]	15^{c}	20^{bc}
	Delta Proteobacteria	*Myxococcales*	*Polyangiaceae*	*Sorangium*	*Sorangium cellulosum*[18]	15^{c}	10^{c}
	Betaproteobacteria	*Nitrosomonadales*	*Nitrosomonadaceae*	*Nitrosomonas*	*Nitrosomonas nitrosa*[19]	7^{b}	9^{b}
	Deltaproteobacteria	*Syntrophobacterales*	*Syntrophobacteraceae*	*Desulfovirga*	*Desulfovirga adipica*[20]	2^{c}	6^{c}
	Gammaproteobacteria	*Xanthomonadales*	*Xanthomonadaceae*	*Lysobacter*	*Lysobacter brunescens*[21]	8^{c}	5^{c}
	Deltaproteobacteria	*Bdellovibrionales*	*Bacteriovoracaceae*	*Peredibacter*	*Peredibacter starrii*	1^{c}	3^{c}
Cyanobacteria	*Cyanophyceae*	*Oscillatoriales*	*Oscillatoriaceae*	*Oscillatoria*	*Oscillatoria acuminata*[22]	1^{b}	6^{b}
Bacteroidetes	*Sphingobacteriia*	*Sphingobacteriales*	*Chitinophagaceae*	*Parasegetibacter*	*Parasegitibacter luojiensis*[23]	2^{b}	1^{b}
Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinacaea*	*Methanosarcina*	*Methanosarcina mazei*[24]	0^{b}	0^{b}

Values are mean of 3 replicates. Values followed by different alphabets in the rows are significantly different at $P < 0.05$ using Least Significant Different (LSD) test. Numbers written in superscripts form are for showing the possible roles of species. +, Positive; -, Negative; NA, No data available

[1] Possess pathogenic properties, found from nonhealing cutaneous wound in a cat (Wells et al., 2018)

[2] Can convert nitrate to nitrite, can hydrolyse starch (Tamura et al., 2001)

[3] Can produce cell-wall-degrading enzymes (like β-1,3-Glucanase), for the management of crop diseases (like Pythium) (El-Tarabily, 2006).

[4] Ability to reduce nitrate to nitrite, has salt tolerance capacity (upto 3 % NaCl) (Tiago et al., 2006)

[5] possess properties for biodegradation and bioremediation of pollutants. It can also degrade polyethylene (Guevara et al., 2019; Orr et al., 2004)

[6] a selenate- and arsenate-reducing bacterium (Yamamura et al., 2007)

[7] has the ability to oxidize NO_2^- (Nitrite) to NO_3^- (Nitrate) –important role in nitrification and denitrification process (Whalen and Sampedro, 2009)

[8] possess plant growth promoting activity (Wang et al., 2016)

[9] endospore-forming, halotolerant bacteria, able to reduce nitrate to nitrite and nitrite to N_2, also catalase and oxidase positive (Raats and Halpern, 2007)

[10] possess the arsenic tolerant capacity and able to remediate arsenic from the contaminated soil (Achal et al., 2012)

[11] industrially important bacterium due to production of antibiotics such as fosfomycin and bacitracin. Also considered plant growth-promoting rhizobacterium (Lekota et al., 2018)

[12] have a positive effect on control of root-knot nematode (Podestá et al., 2013)

[13] Human pathogenic bacterium (Cautivo et al., 2020)

[14] shows excellent control of Plant-parasitic nematodes and has been produced as a commercial nematicide (Geng et al., 2016)

[15] Has bioremediation capacity, can transform arsenic from arsenic contaminated soil (Jebeli et al., 2017)

[16] Contains pathogenic property (Jin et al., 2017)

[17] an ammonia oxidizing bacteria, comes under nitrifying bacteria. Has monooxygenase activity (Harms et al., 1976)

[18] producer of secondary fungicides and bactericides that reduce competition in soil environments. Produce compounds that are antifungal, antibacterial, and antibiotic resistant (Pradella et al., 2002)

[19] an ammonia oxidizing bacteria (Garrity and others, 2005)

[20] an adipate-degrading, sulfate-reducing bacterium (Tanaka et al., 2000)

[21] possess antibacterial property (Ling et al., 2019)

[22] Can produce saxitoxin, a neurotoxin (Mohamed and Al-Shehri, 2015)

[23] Positive for having acid and alkaline phosphatase activity, an important soil enzymes for phosphate availability (Zhang et al., 2009)

[24] has an ability to digest organic waste (Tatton et al., 1989)

3.7. Effect of KSWE on soil enzymes

The influence of KSWE on five different soil enzymes at the harvest of maize crop is shown in Table 7. Compared to the control (T3), alkaline and acid phosphomonoesterases, aryl sulphatase, glycosidase, and FDA hydrolysis had significantly higher activities due to the application of KSWE (T4).

Table 7

Influence of KSWE on soil enzymatic properties applied at V5,10,15 growth stages of maize plant under drought at same stages. Values are mean of 3 replicates. Values followed by different alphabets in the columns are significantly different at $P < 0.05$ at Tukey's HSD.

Treatments	Aryl sulphatase (µg of nitrophenol released h^{-1} g^{-1} dry soil)	Fluorescein Di-acetate (FDA) (µg of fluorescein g^{-1} dry soil)	Alkaline phosphatase	Acid phosphatase	Glycosidase
			µg of nitrophenol released h^{-1} g^{-1} dry soil		
T3- V5,10,15 water spray	7.71 ± 1.01[b]	54.53 ± 11.14[b]	327.51 ± 34.58[b]	311.15 ± 31.61[b]	266.76 ± 38.14[b]
T4- V5,10,15 KSWE	12.04 ± 1.01[a]	80.95 ± 9.30[a]	484.96 ± 15.84[a]	408.90 ± 46.63[a]	387.40 ± 57.21[a]

3.8. Effect on crop yield

The cob yield of maize plants was significantly altered due to the treatments (Table 2). Significantly higher cob yield was observed in the treatments where KSWE was applied once or thrice along with drought stress compared to their respective controls. The highest cob yield was observed under the treatment receiving normal irrigation along with the KSWE application on a daily basis.

Discussion

From the results of the initial studies on a few PGPRs and total soil bacterial CFUs under different soil moisture regimes, it was apparent that KSWE is likely to have a positive influence on soil bacteria under drought conditions. Further, our study showed that on an average, 23% of the spray volume of KSWE falls to the ground either as a drift or from the leaves as a drip (excess spray accumulating on the leaf surface drips to the ground), considering foliar spraying at V5, V10 and V15 stages of maize (Table 1). Hence KSWE was conjectured to have a considerable effect on soil bacterial community structure and function. In addition to direct effect, KSWE may also be indirectly contributing to growth and productivity of crop by modulating the soil ecology by effecting a desirable bacterial shift under stress conditions, which formed the hypothesis of this study. The study showed that KSWE helps the soil bacteria to recover from the negative impact of drought by rebuilding the affected soil microbial population, especially the species having functional importance in soil nutrient transformation and cycling.

This study characterized the effect of week-long drought stress subjected once or thrice during the critical stages of maize crop with or without the use of KSWE on soil bacterial community and compared it with the bacterial community present in normally irrigated soil. A detailed investigation carried out on soil collected at harvest revealed propensity towards a major shift in soil bacterial communities due to drought when compared to normally irrigated soil. This tendency to restructure the microbiome by drought was thwarted by KSWE application when applied three times at the critical crop stages. It was evident that soil moisture stress, when subjected early at the V5 stage, continued to have a detrimental effect on the relative abundance of soil bacteria till harvest in spite of resuming normal watering thereafter. Application of KSWE once at V5 stage probably did not help the abundance to recover till the end of the cropping season, which happened in a case where KSWE was applied thrice at V5, 10 and 15 stages. Bacteria adopt various strategies to ward off stress, for example by producing stress-resistant spores (some members of *Firmicutes*, *Actinobacteria*), biofilm formation, osmoprotectant production (Santos-Medellin et al. 2017), alterations in gene expression and protein activity (Boor 2006) leading to quiescence or dormancy. From the results, it is conjectured that repeated KSWE application three times helped such bacteria to break quiescence/dormancy and regain their growth potential. Notably, KSWE was found to contain quaternary ammonium compounds like carnitine (unpublished) and betaines (Mondal et al. 2015) which have been reported for their osmoprotectant and growth rate stimulating activity in some microorganisms (Kleber 1997). The effect of these compounds in KSWE might explain the improvement in the number of OTUs upon its application under drought stress. The restructured microbial community could also have been due to the crop response to soil moisture stress that could have modulated diverse plant processes such as quality and quantity of root exudates (Song et al. 2012), leaf fall on the ground and their subsequent decomposition, or changes in the microhabitat due to differential above- and below-ground plant growth to which the native microbiome reacted. Further, a significant role of antioxidant activity in leaves for achieving high productivity of maize and drought resistance was earlier reported in our findings (Trivedi et al. 2018a); the same was also found true in the present experiment (data not shown here as not in the scope of the present manuscript). In brief, KSWE significantly enhanced the activity of the stress enzymes in leaves, namely, catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) when applied at V5,10,15 (T4), compared to its controls under drought.

The results of our present study also demonstrated that the KSWE application had no detrimental effect on the dominant soil bacterial microbiome, except in the case of *Chloroflexi*, even when applied on a daily basis under normally irrigated conditions. Chuah et al. (2017) also reported zero inhibition in bacterial growth of some species upon application of the crude extract of *K. alvarezii*. Our results are also in conformity to the reported observation that the relative abundance of *Chloroflexi* increases under moisture stress conditions (Santos-Medellin et al. 2017). *Chloroflexi* is composed of monoderm bacteria with thick cell walls that have better soil moisture stress resisting abilities (Lennon et al. 2012) because of which they would continue to thrive better compared to others and would be in a greater proportion under moisture stressed soils as was also observed in this study. The observation that the nearly doubled relative abundance of *Chloroflexi* in soil subjected to stress (T3) was brought down to the level present in the normal irrigated soil by KSWE application, supported the role of KSWE in eliciting stress response (Fig. 3).

The overall response of the bacterial community to soil moisture stress and KSWE was more or less consistent across all the taxonomic levels. At phylum level, the response to KSWE under stress (T4), when compared to its corresponding control (T3) was primarily driven by a significant enrichment of multiple phyla such as *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Planctomycetes*, *Bacteroidetes* and *Verrucomicrobia* (Fig. 4), all of whose abundances were diminished under stress when compared to normally irrigated conditions. The enriched relative abundance of these phyla brought about by KSWE under stress was at par to that under the normal irrigated condition and this observation was confirmed by beta diversity analysis depicted by a phylogenetic tree and PCA analysis, wherein T4 and T5 clustered more or less together, while T3 clustered separately from these two treatments. Within the *Firmicutes*, the most enriched OTUs in T4 compared to its control (T3) were mainly from the genera *Clostridium*, *Bacillus*, and *Alicyclobacillus*, all of which were endospore-forming bacteria. The higher relative abundance of several OTUs classified as *Proteobacteria* due to T4 treatment (compared to T3) was mainly due to the genus *Steriodobacter* and *Balneimonas* falling under *Gamma-* and *Alpha -proteobacteria* classes, respectively. The genera most affected by soil moisture stress were *Anaerolinea* and *Nitrospira* falling under the phylum *Chloroflexi* and *Nitrospirae*, respectively. The relative abundance of both these genera under drought was increased due to KSWE when applied three times. Many other genera playing an important role in nutrient cycling in soil were also found to be beneficially influenced by KSWE when applied three times under stress. For example, compared to T3, the relative abundance of the genus *Rubrobacter* under the phylum *Actinobacteria* —a thermophilic bacteria capable of degrading xylan, chitin, cellulose and hemicellulose and having an important role in organic matter turnover and C cycling — was significantly higher and may thus influence nutrient availability to plants for better growth and yield. The phylum *Actinobacteria* is also associated with the production of bioactive compounds and plant growth promoters which might influence the growth and yield of crops. Similarly, various genera instrumental in ammonia oxidation (*Nitrosomonas*, *Nitrosovibrio*, and *Nitrospira*) also were beneficially increased by KSWE under severe stress conditions compared to its respective control and their abundance levels were raised at par to that under normal irrigated condition (Appendix S7). The other taxons

important for N cycling such as the phyla *Chlorobi* and those important for potassium solubilization such as *Paenibacillus* genus also followed the same trend (data not shown). The microbial communities are expected to play a crucial role in biochemical activities in the soil and thus the soil enzymes are crucial in C, P and S cycling. They can be used as soil quality indicators since they have more sensitivity to the changes in soil properties (Anand et al. 2015). The enrichment of species having functional roles such as in nutrient transformation and heavy metal tolerance (table 5 and 6) clearly brings out the beneficial role KSWE plays in soil for the benefit of crop growth and productivity. In agreement, the activities of various soil enzymes involved in P (acid and alkaline phosphatases), S (aryl sulphatase) and C (glycosidase) cycling were higher in T4 compared to the T3 treatment.

The observed KSWE-mediated changes in the soil bacterial communities and soil enzyme activities were also associated with higher crop yields under soil moisture stress conditions. Thus, this study provides a comprehensive understanding of how the KSWE could impart stress tolerance not only to the host plants but also was beneficial in providing a conducive soil bacterial microbiome. Further elaborate field studies are warranted to understand soil-plant-microbe interactions.

Conclusion

This study provided a comprehensive understanding of how the KSWE could impart stress tolerance not only to the host plants but also to the extent to which it enriched the soil bacterial microbiome elucidated to species level under stress conditions. The essence of the study was also to show that the KSWE application had no deleterious effect on the soil bacterial community and that the consequential beneficial effects may contribute towards improvement in the crop yields by improving nutrient cycling in soil.

Declarations

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Code availability: Not applicable

Authors' contributions: KT performed experimental work, contributed to data acquisition, its analysis and interpretation. RK, GB and DK contributed during data acquisition and drafting of the article. KGV contributed to data acquisition, drafting of manuscript and reviewed the manuscript critically for important intellectual content. AG designed the experiment, coordinated the work, interpreted data and critically reviewed the manuscript. All the authors have read and approved the final manuscript.

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Figures

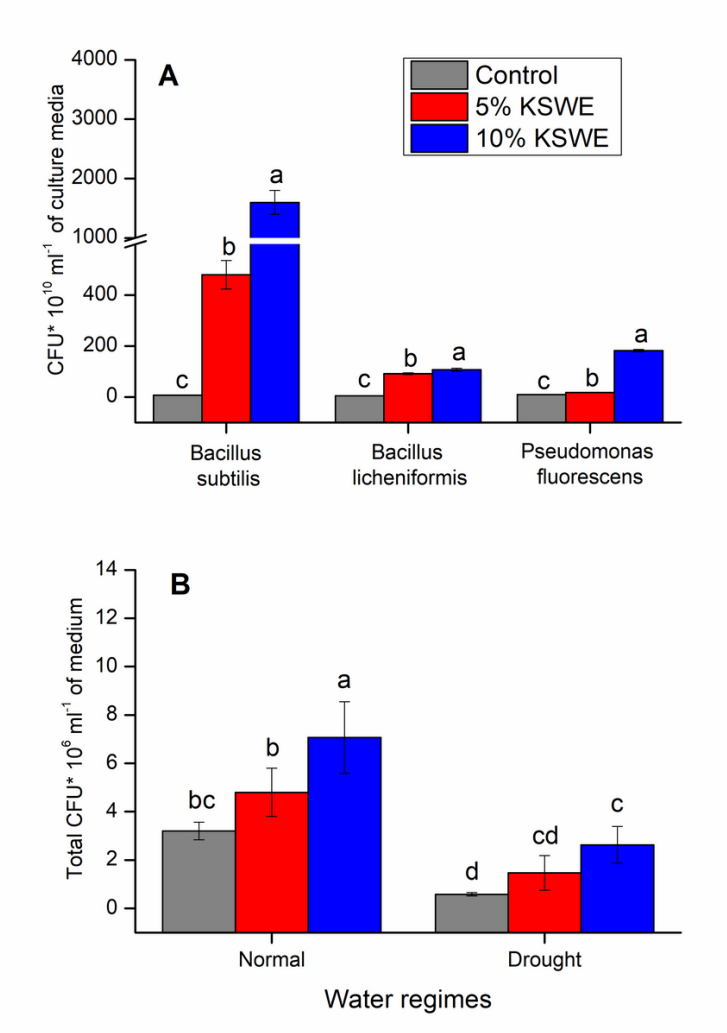

Figure 1

A) Change in colony forming units (CFU) of different plant growth promoting bacteria in their bacterial cultures upon application of two different concentrations of KSWE (5 and 10%) along with control. Bars followed by different alphabets within the bacterial species are significantly different at $P < 0.05$ using Tukey's HSD. B) Change in total CFU of soil upon application of 5 and 10% KSWE as a drench under normal as well as drought conditions. Bars followed by different alphabets are significantly different at $P < 0.05$ using Tukey's HSD.

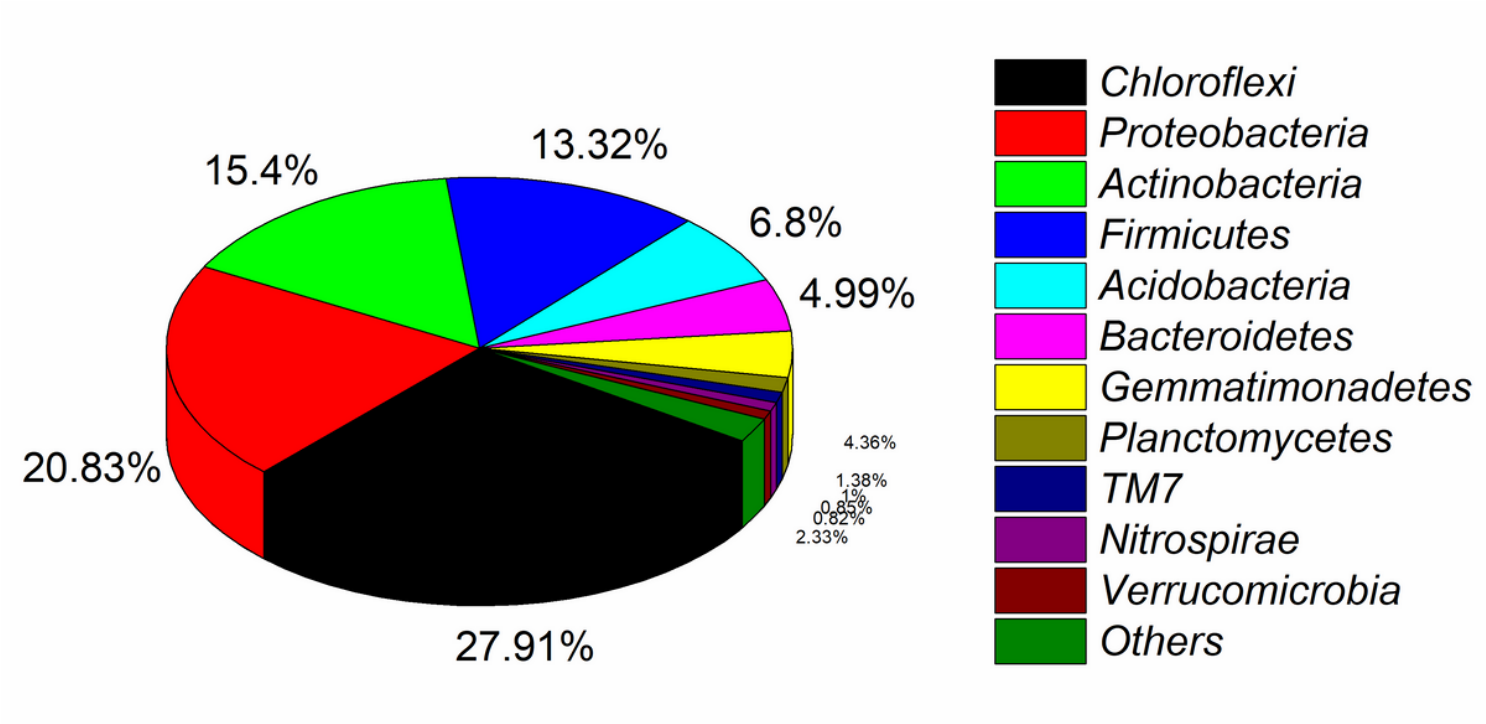

Figure 2

Top 12 phylum categories from all five treatments.

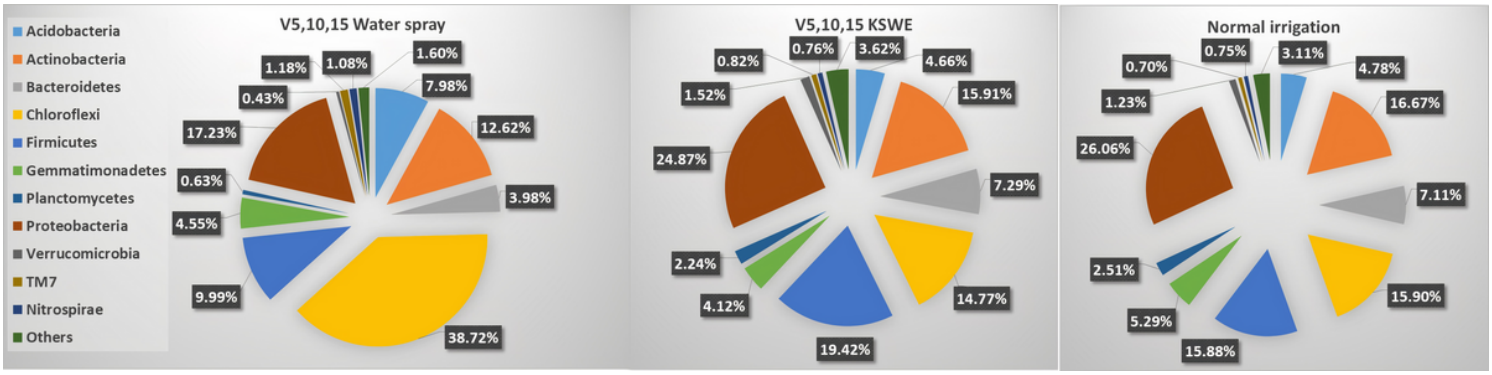

Figure 3

Relative abundance shift of top 12 phylum categories observed among soils treated with normal irrigation, drought stress at V5,10,15 and drought stress along with KSWE application at V5,10,15 stages.

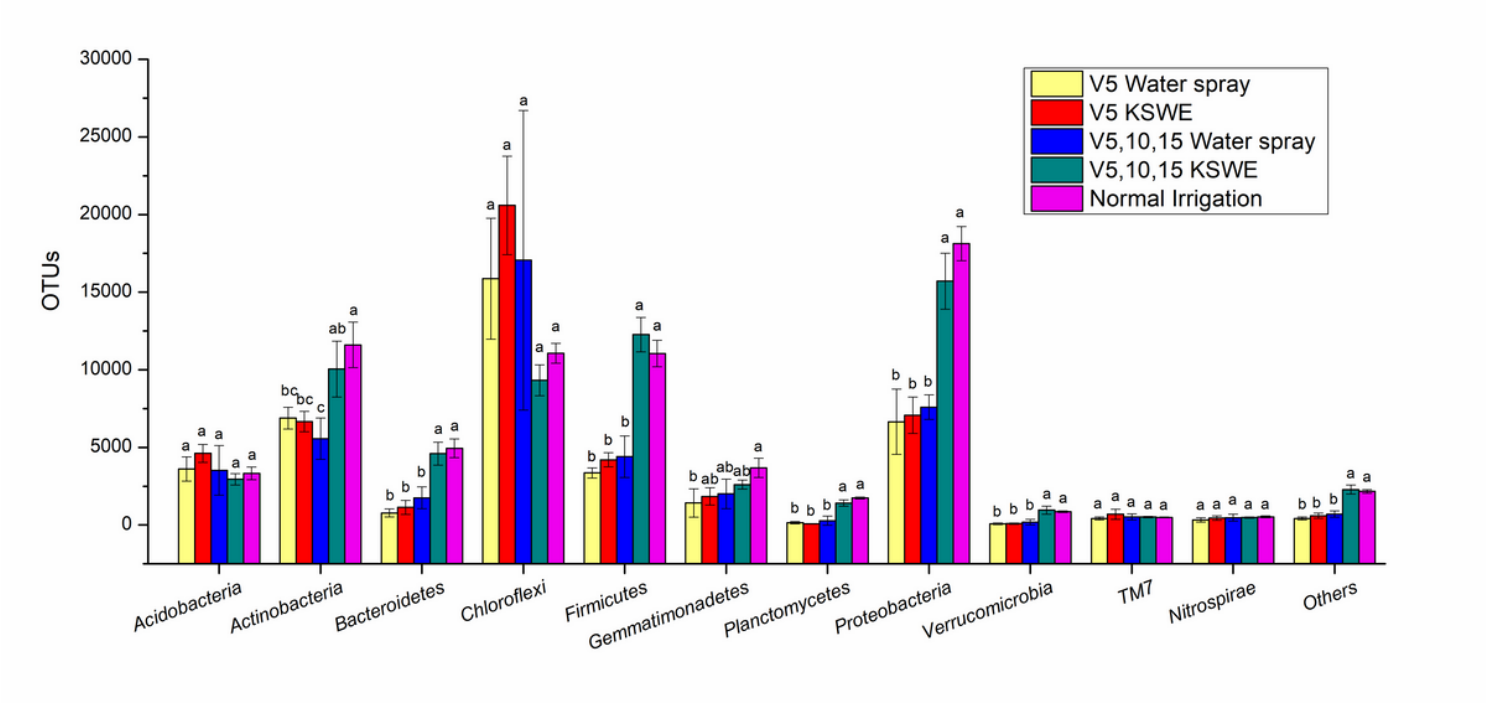

Figure 4

Taxonomy classification of OTUs at phylum level. Only top 12 enriched phylum categories are shown. Values are mean of 3 replicates. Bars followed by different alphabets within the treatment are significantly different at $P < 0.05$ using Tukey's HSD.

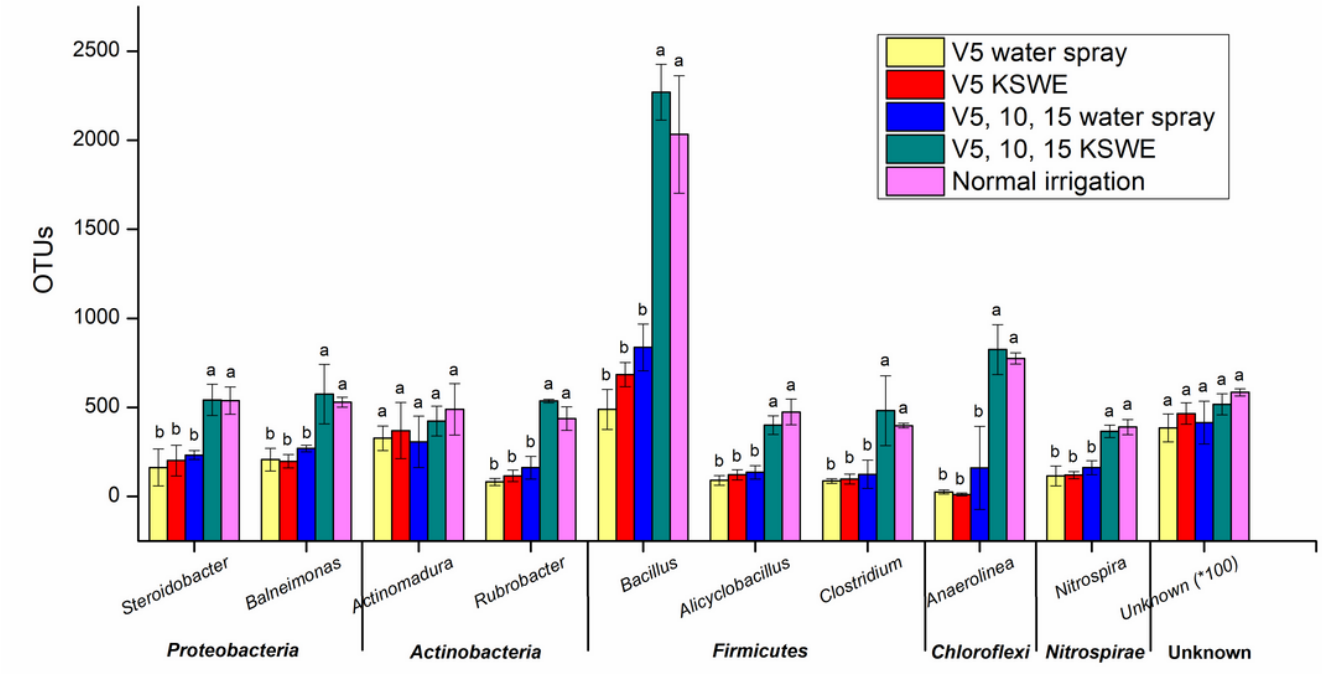

Figure 5

Taxonomy classification of OTUs at genus level. Only top 10 enriched genus categories with their relevant phylum in bold are shown. Values are mean of 3 replicates. Bars followed by different alphabets within the treatment are significantly different at $P < 0.05$ using Tukey's HSD.

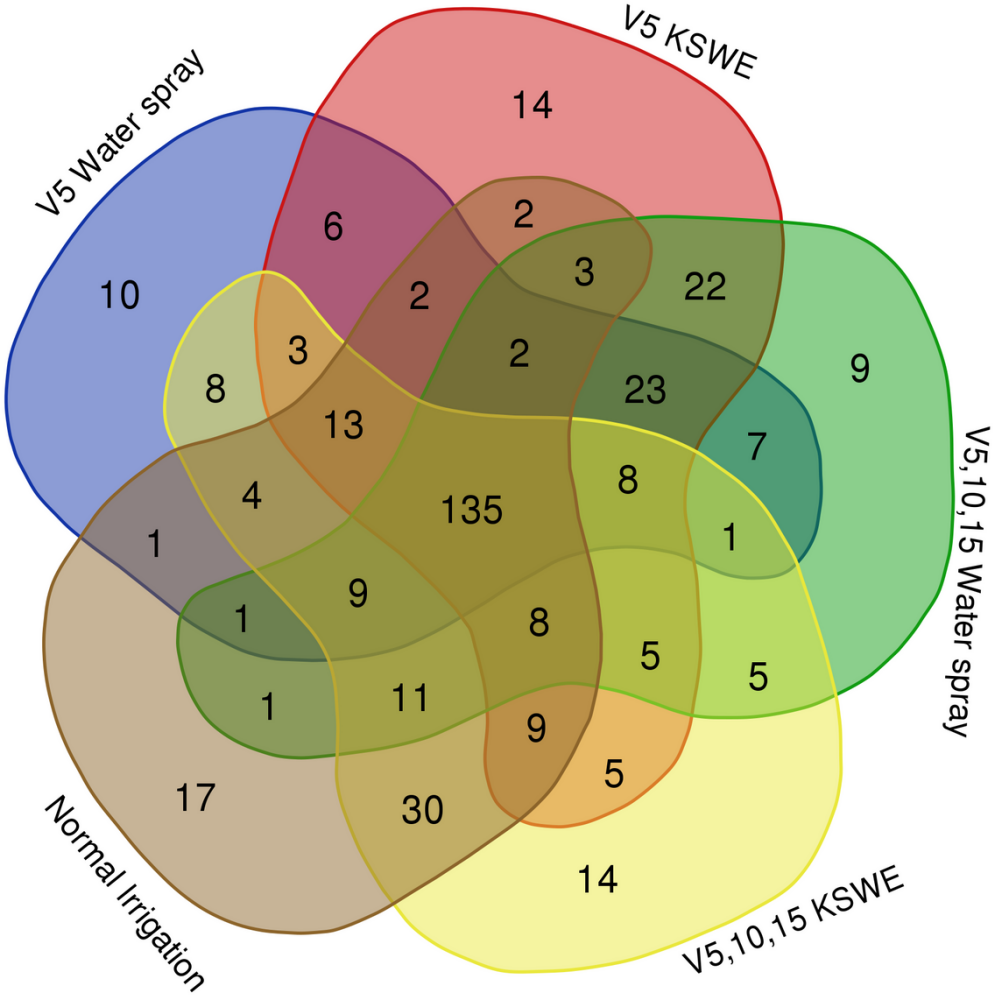

Figure 6

Venn diagram of all five treatments to observe the number of unique and shared species present among them.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AppendixS1Meteorologicaldata.docx
- AppendixS2Heatmaps.docx
- AppendixS3Rarefactioncurves.docx
- AppendixS4Phylogenetictree.docx
- AppendixS5PCAofphylumsandgenus.docx
- AppendixS6Biplots.docx
- AppendixS7Relativeabundance.docx