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Seasonal Variation in the Rhizospheric Microbial Diversity of the Weedy Plants

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Abstract:

Plant-microbe interaction forms the intrinsic part of our ecosystem. This interaction is responsible for remediating contaminants, sequestration of carbon, plant disease control and plant growth promotion. Phytoremediation capabilities can be determined by thriving microbial communities and variation in their species composition. The composition of soil microbiome is affected by various factors such as environment, climate and plant genotype. In the present study, we have assessed the seasonal variation in the bacterial community structure in the rhizospheric microbiome of weedy plants, *Acorus calamus, Typha latifolia*, and *Phragmites karka* using T-RFLP. The bacterial phyla dominating the summer season belonged to *Proteobacteria, Bacteriodetes, Firmicutes* were seen to abundant in *P.karka* followed by *A.calamus* and *T.latifolia*. During the winter season the bacterial phyla that dominated belonged to the phylum *Bacetriodetes, Proteobacteria, Firmicutes* were found abundant in *P. karka* followed by *A. calamus* and *T. latifolia*. Diversity indices of the bacterial community were assessed. The result of this study shows the presence of seasonal variation in bacterial phyla which can act as the potential candidates for the remediation process.

Keywords:
Phytoremediation, Rhizosphere, *Acorus calamus, Typha latifolia, Phragmites karka.*

INTRODUCTION

The presence of microbial communities in the functional wetlands play a major part in the elimination of the contaminants as the plant-microorganism interaction in the rhizosphere plays an important role in nutrient cycling, carbon sequestration in the natural ecosystem and ecosystem functioning. Microbes form an integral part of the soil ecosystem. They form an essential component of biogeochemical and nutrient cycles in the environment. Microbes are available in different ecological niches, however, bacterial population found in extreme ecosystems shows variation from one another. Microbes are compelling as well as are responsive in nature to the surrounding ecosystem affected by factors such as nutrients, toxicants, temperature, pH, and moisture. We can exploit this property of these soil microbes, making them act as the bioindicator of changing environmental parameters. Various studies have been conducted on the soil bacterial community and represent their dynamic nature in correlation to the environmental parameters. The composition of the microbial community composition present in the rhizoplane is majorly determined by the plant root exudates.

The composition of the root exudates differs among various plant species, thus playing an influential role in the comparative abundance of microorganisms colonizing the root surface of the plant. Thus, the phytoremediation capability will be determined majorly by the abundance of microbial communities and variation in species composition [1]. Microorganisms are sensitive in nature towards availability of nutrients thus they also display a shift in their community structure in response to the seasonal cycles. The increasing industrialization and the disposal of hazardous contaminants into the environment have majorly affected the native bacterial community structure[2].

During various studies conducted on the wetland plant species, rhizospheric bacterial community composition was characterized by means of culture-dependent or culture-independent techniques [3]. Whereas there are few reports on the rhizospheric microbial communities of *Acorus calamus*, *Typha latifolia* and *Phragmites karka* and nothing significant are known regarding the epiphytic bacteria associated with these plants.

Physicochemical parameters of the sewage water which is still majorly used in the irrigation shows increased BOD and decreased DO level, that occurs due to the contamination of heavy metals [4] and pesticides[5,6]. Heavy metals cannot be degraded in the environment; they tend to persist in the organisms and are harmful to them. The concentration of heavy metals and pesticides present in the sewage water was found to vary seasonally due to increasing temperature of water leads to increasing concentration of heavy metal during the summer season[7,8,9]. Heavy metals tend to affect the structural as well as the biological functioning of biomolecules[10]. Heavy metals are known to interfere with synthesis along with the metabolism of hormones[11,15]. Thus, it is important to understand the ecological dynamics taking place in the bacterial community present in the rhizospheric zone of the wetland plants.

In the present study, the microbial population in the rhizospheric zone of the wetland plants was identified by using Terminal restriction fragment length polymorphism (T-RFLP)[13]. In the case of T-RFLP, the sample DNA is subjected to universal 16S r DNA primers for their amplification where one of the primers is fluorescently labelled. The fluorescently labelled terminal restriction fragments (T – RFs) were further subjected to restriction enzymes and results thus obtained were analysed using a DNA sequencer. Then the sizes of T- RFs along with their amount are calculated. Differentiating on the basis of peak size and peak area, also correlating these patterns to environmental factors *in – silico* we can analyse the microbial community structure [14].

MATERIALS AND METHODS

Site description and Soil Sampling

In order to compare the rhizospheric microbial diversity among three wetland plants: *Acorus calamus, Typha latifolia and Phragmites karka*, sampling was done twice in a year, in November 2013 (winter season) and in May 2014 (Summer season). The root samples of *Acorus calamus*, *Typha latifolia*, *Phragmites karka* were collected from the established wetland mesocosms which were maintained at IARI, Delhi. Root length of 15 cm sections was collected carefully from the plants. Subsamples of the roots were collected from 3 to 4 separate plants present across the sampling site and were merged to form a single sample. The collected samples were kept in sterile sampling bags. They were transported to the lab in the ice box, where the temperature inside did not exceed 6^{o}C. Samples were stored in 4^{o}C in the lab and were preceded for analysis the same day. Water sampling was done from the sewage water drain of IARI. The water sample was collected in triplicate in 250 ml pre-cleaned oven dried sampling bottles and was sealed with the screw cap. The samples were placed in 4^{o}C and were tested within 24 hours of sampling.

Water physiochemical properties

Physiochemical properties were done on the water sample collected from the sewage water drain of IARI. The pH and temperature were analyzed using a waterproof pH tester "pHTestr 30" (Eutech

Instruments). Total heavy metal content was determined using AAS (AA 8000, PG Instruments, UK) by EPA's acid digestion procedure [12]. For the measurement of the Electrical conductivity, Electrical conductivity meter (Corning) was used. In order to measure the Dissolved Oxygen, Do Pen (SPER SCIENTIFIC) was used. The TDS was measured using the TDS meter (Corning).

Rhizospheric microbial community analysis

The DNA was extracted in triplicates, from the 500 µl of bacterial cell suspension, using the DNA extraction kit (Mo Bio) as per the manufacturer's procedure. Extracted DNA from the triplicate root samples were pooled together and then checked on 0.8 % agarose gel and quantified at 260/280 nm on NanoDrop (ND-1000, Spectrophotometer, Thermo Fisher Scientific, DE, USA).

Amplification and T-RFLP of 16S rRNA gene

The amplification of the 16S rRNA gene was done from 1 µl of 50 ng of extracted soil DNA on PCR Thermocycler (BIO-RAD, USA) with a total volume of 50 µl by using 1 µM concentrations of the universal primers, i.e., FAM™ labelled 8F (5′ – AGAGTTTGATCCTGGCTCAG-3′) and 928R (5'CCGTCAATTCCTTTGAGTTT-3') (Sigma). The PCR mix (25µl) included 25ng of template, 1XPCR buffer, 200µM each dNTP (Genei™, Bangalore), primer 1µM each, and Taq polymerase 1.5U (Genei™, Bangalore). Following were the PCR conditions: the first step is the primary denaturation at 94^{o}C (5min), followed by 30 cycles of denaturation at 94^{o}C (1min), then annealing at 55^{o}C for 30 sec takes place, extension at 72^{o}C (2 min), followed by the last extension step at 72^{o}C takes place for 7 min and finally the end step is held at 4^{o}C. The amplified products were visualized the gel documentation system (Gel Doc™ XR+BIO RAD, USA) on 0.8% agarose gel. Bands were excised and eluted using PCR cleanup kit (Wizard® SV Gel and PCR Cleanup System, Promega, Madison WI, USA). 100 ng of eluted DNA samples were digested using 2.5 U of three different restriction enzymes, i.e., AluI [AG^CT], MspI [C^CGG], and HaeIII [GG^CC] at 37 °C for 12 h. The digested products were then sequenced from SCI genome laboratories, Cochin, India. T-RFLP profiles obtained were visualized using GeneMapper® software (version 4.0. Applied Biosystems).

T-RFLP analysis of 16S rRNA gene

T-RFLP profile data of the 16S gene consist of size (length) in base pair, peak height, peak area, and data point for each T-RF in the sample. In order to avoid detection of primers, fragments having a size less than 25 bp were excluded. For each T-RF in the respective samples, percentage relative abundance was calculated by the ratio of peak area of each peak to the total peak area of a sample. Fragment Sorter 5.0 (FragSort 5.0, Ohio State University, USA) was used to identify the specific rhizospheric microorganisms in a community based on their T-RFs length. T-RFs data in the form of .csv files was uploaded in the software. T-RFs data were arranged according to T-RFs size and relative percentage abundance of peak area. The results thus obtained from this software are a list of microorganisms along with their T-RF sizes that correlate with collective experimentally generated T-RFLP profiles. The output thus obtained was sorted on microorganism's name.

Statistical analysis

Level of significance in all the above-studied parameters was assessed using two-way analysis of variance (ANOVA) followed by the Tukey's test ($P < 0.001$, Sigma plot version 13.0). Two-way ANOVA was performed in order to observe the impact of heavy metal concentration as well as climatic conditions on the soil microbial diversity.

In order to compare the rhizospheric microbial species diversity among the samples, the Shannon–Wiener diversity index was calculated as $H' = -\sum (Ni/N) \times Ln (Ni/N)$. Ni = number of individuals of species "i" or % peak area of species "i" and N = total number of individuals of all species or the total sum of the peak area of an individual sample. The study of diversity index helps us to analyse the relative intricacy present among communities and helps us to assess the completeness of sampling [15, 16].

On the basis of OTU's rarefaction curve was also constructed for both the seasons, they help to analyse the calculation of species richness for a given number of individual samples. Using MVSP 3.1 statistical package, UPGMA and PCA clustering were conducted in order to analyse the rhizospheric microbial community similarity [17]. On the basis of Jaccard measure of similarity the T- RF's profile that is similar to one another is grouped together using the UPGMA clustering. Using these statistical analyses we can predispose the relationship between microbial OTUs (operational taxonomic units) distribution and its community composition as a function of the seasons, geographic origin, or habitat structure [18, 25].

RESULTS

Physiochemical Properties

The collected water sample from the sewage drain present in IARI was further subjected to analysis of its water quality parameters such as pH, Electrical conductivity (EC), Temperature, Dissolved oxygen (DO) and Total Dissolved solids (TDS). The mean value and standard deviation of the physico – chemical properties of water are depicted in the Table 1. The pH of the sample was calculated to be 7.02 ± 0.03 along with the temperature 23.1 ± 0.35°C during winters whereas the pH was recorded to be 8.02 ± 0.17 during summers along with temperature at 35.1 ± 0.35°C. EC was seen to be 2.45 ± 0.05 mS cm $^{-1}$ during winters and 1.45 ± 0.15 mS cm^{-1} during the summer season, whereas the TDS was evaluated to be 976 ± 2.8 mg $^{-1}$ during the winter season it was measured to be 431± 7.8 mg $^{-1}$ during the summer season. DO was measured to be 2.66 ± 0.06 mg $^{-1}$ during the winter season whereas it was measured 1.58 ± 0.06 mg $^{-1}$ during the summer season.

Table 1: Diversity indices of the root epiphytic bacteria population during seasonal variation in *A. calamus*, *T. latifolia* and *P. karka*

Winter Season			**Alpha Diversity**	**Summer Season**		
Acorus calamus	***Typha latifolia***	***Phragmites karka***		***Acorus calamus***	***Typha latifolia***	***Phragmites karka***
0.6952	0.6203	0.7108	Simpson's Diversity Index (1-D)	0.5859	0.5808	0.5751

The total heavy metal content of the sewage water was observed and tabulated in Table 2. Metals such as Cadmium, Copper, Lead and Chromium were found to be significantly higher than the permissible limits in both the seasons.

Table 2: Heavy metal Concentration (ppm) in the sewage water collected at IARI, New Delhi, INDIA

Heavy Metals	**Permissible limits (mg/l)**	**Concentration during Summer season (mg/l)**	**Concentration during Winter season (mg/l)**
Beryllium (Be)	0.1	0.0004	0.0005
Aluminium (Al)	5	0.029	
Tin (Sn)	3.2	0.036	0.048
Lead (Pb)	0.01	0.112	0.1
Arsenic (As)	0.1	0.0358	0.106
Chromium (Cr)	0.07	2.6	0.0337
Cobalt (Co)	0.2	0.009	1.57

Nickel (Ni)	0.04	0.005	0.12
Copper (Cu)	0.05	3.633	0.016
Zinc (Zn)	2	0.914	1.453
Cadmium (Cd)	0.1	0.0016	0.816
Silver (Ag)	3.5	0.0001	0.00119
Vanadium (V)	0.01	0.01	0.0001

T-RFLP analysis of 16S rRNA gene

In the present study, microbial diversity and their structural dynamics were observed by using 16S rRNA gene in wetland plants (*Acorus calamus, Typha latifolia and Phragmites karka*) under two different seasons (winter and summer) with the help of T- RFLP technique. FragSort analysis of sequencing result for each plant sample during different season was done and the percentage relative abundance was calculated. On the basis of FragSort analysis; *Phragmites karka* dominated the diversity of bacteria followed by *Typha latifolia* and *Acorus calamus* during both the seasons but there was a difference in species richness. Bacterial phylum that dominated the summer season belonged to phyla *Bacteriodetes*, followed by *Proteobacteria* and *Firmicutes* (Fig. 1)[19]. Other bacterial phyla were also observed but were not seen to be present in all the three plants; *Fusobacteria, Actinobacteria, Acidobacteria* were present both in *P. karka* and *A. calamus* but was absent in *T. latifolia*. Phylum *Chloroflexi* was the only exception that was present in *P. karka* but was absent from rhizospheric zone of *A. calamus* and *P. karka*. During the winter season, the bacterial phyla that dominated belonged to *Proteobacteria* followed by *Bacteriodetes* and *Firmicutes* (Fig. 2). Other bacterial groups belonged to phyla *Actinobacteria* and *Acidobacteria* were present in *P. karka* and *A. calamus* but were absent in *T. latifolia*. Phylum *Chloroflexi* and *Cyanobacteria* were present in low relative abundance but was observed in the rhizospheric zone of all the three plants. Whereas it was seen that phylum *Thermotogae* and *Deferribacteres* were present in low relative abundance but were only present in the plant *P.karka*.

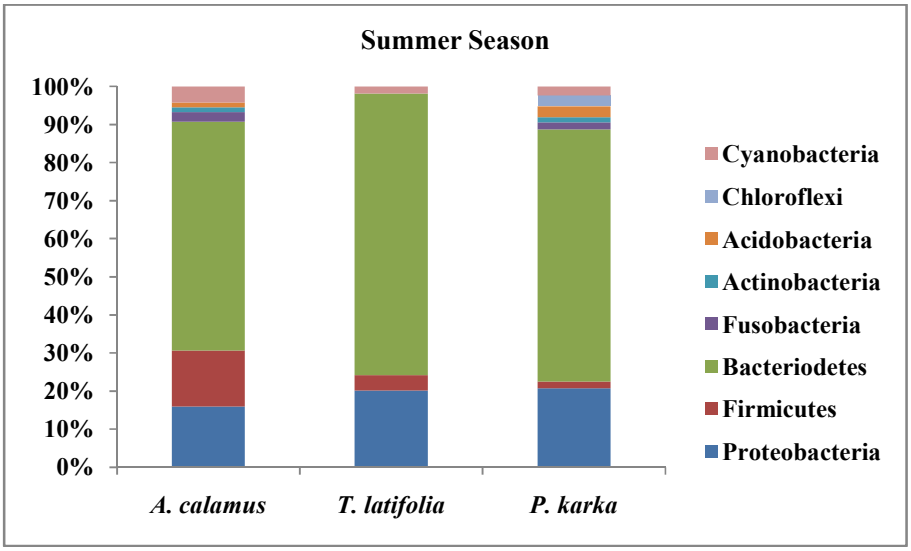

Figure 1: Relative percentage abundance of the dominating bacterial phyla in the wetland plants *A. calamus*, *T. latifolia* and *P. karka* during summer season on the basis of T-RF'S profiles obtained.

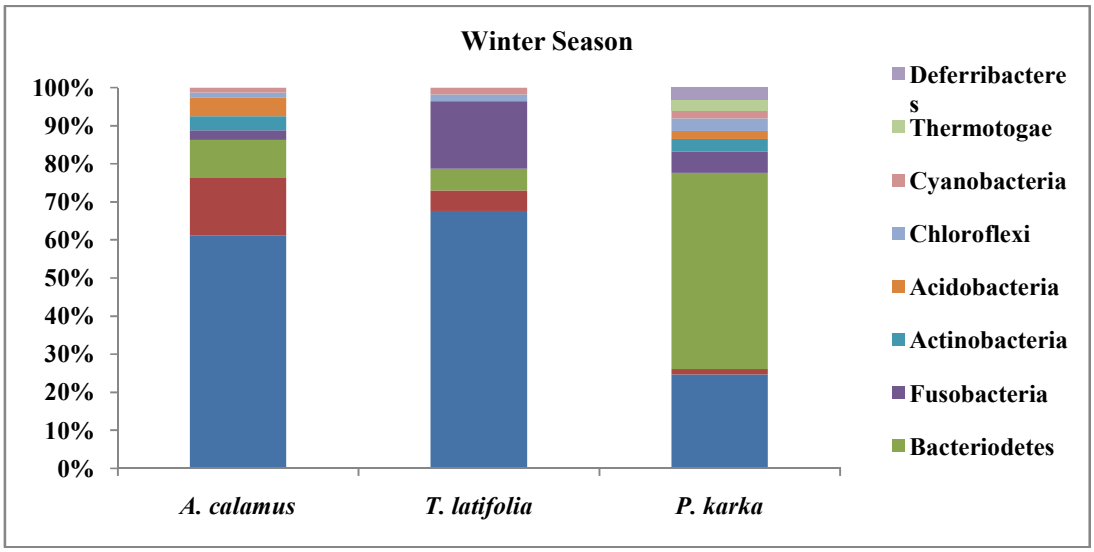

Figure 2: Relative percentage abundance of the dominating bacterial phyla in the wetland plants *A. calamus*, *T. latifolia* and *P. karka* during winter season on the basis of T-RF'S profiles obtained.

In order to understand the temporal variation among the different bacterial groups found in, the rhizospheric zone of the wetland plants during summer and winter season the phyla were hierarchically clustered using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using the Jaccard similarity index. The output result was in the form of dendrogram which illustrates the percentage of similarity among the different microbial groups present in the rhizospheric zone of the wetland plants. Bacterial groups that displayed similar behaviour towards the environmental conditions were grouped together while bacterial groups which displayed dissimilar behaviour were grouped apart from each other. The bacterial phyla during the winter season (Fig. 3) showed that *Actinobacteria* and *Acidobacteria* were grouped together, phylum *Deferribacteres* and *Thermotogae* were grouped together and rest of the bacterial group were grouped together belonged to phylum *Chloroflexi, Fusobacteria Bacteriodetes, Firmicutes* and *Proteobacteria*. During the summer season (Fig. 4); the bacterial phyla *Firmicutes, Cyanobacteria, Proteobacteria* and *Bacteriodetes* were grouped together; whereas rest of the bacterial groups that were grouped together belonged to phylum *Fusobacteria, Actinobacteria* and *Acidobacteria*. Whereas phylum *Chloroflexi* was the only bacterial phyla that were placed alone owing to its low relative abundance as this bacterial group is only present in *P.karka* [23, 24]. In Fig. 5 and 6, the study of the PCA graph during summer season showed that *Typha latifolia* and *Phragmites karka* were placed together as they shared similar microbial diversity whereas *Acorus calamus* had different microbial diversity and was placed separately. During the analysis of the PCA graph microbial diversity showed the dominance of Phyla *Bacteriodetes* followed by *Proteobacteria* and *Firmicutes*. In Fig. 7 and 8, PCA analysis during winter season demonstrated the dominance of phyla *Proteobacteria* followed by *Bacteriodetes* and *Firmicutes*; we can also infer on the basis of PCA analysis that the diversity of bacterial phyla were highest in *Phragmites karka* followed by *Typha latifolia* and *Acorus calamus*[25].

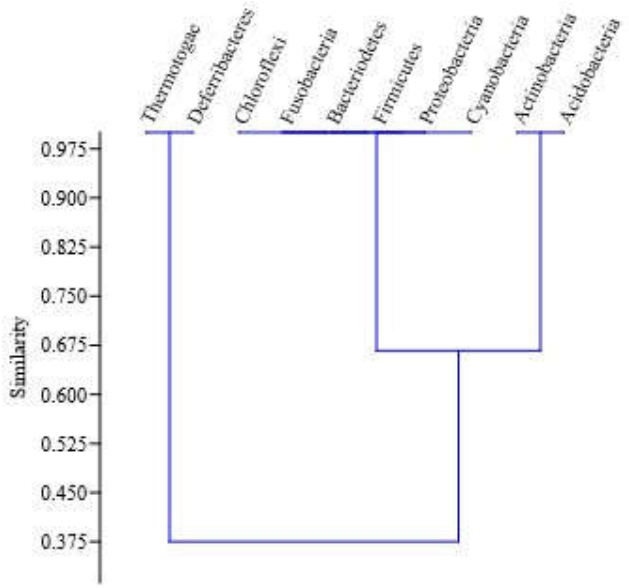

Figure 3: UPGMA dendogram showing coefficient of similarity between the bacterial phyla obtained from the root epiphytic zones from the wetland plants during winter season. Similarity coefficient was calculated by using the Jaccard's index on the basis of their percentage abundance.

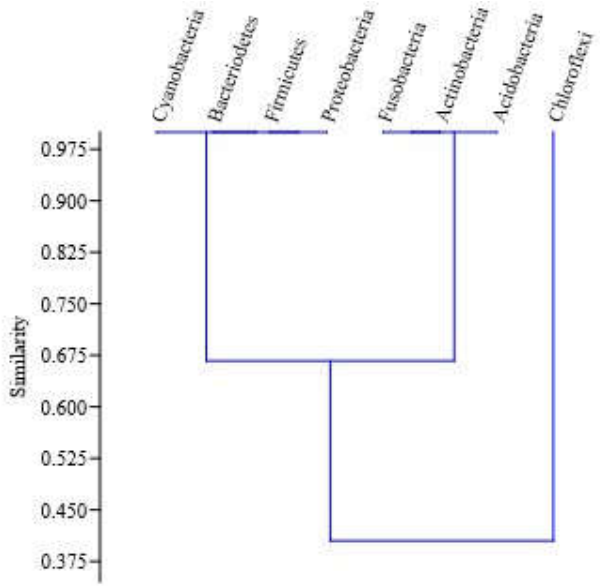

Figure 4: UPGMA dendogram showing coefficient of similarity between the bacterial phyla obtained from the root epiphytic zones from the wetland plants during summer season. Similarity coefficient was calculated by using the Jaccard's index on the basis of their percentage abundance.

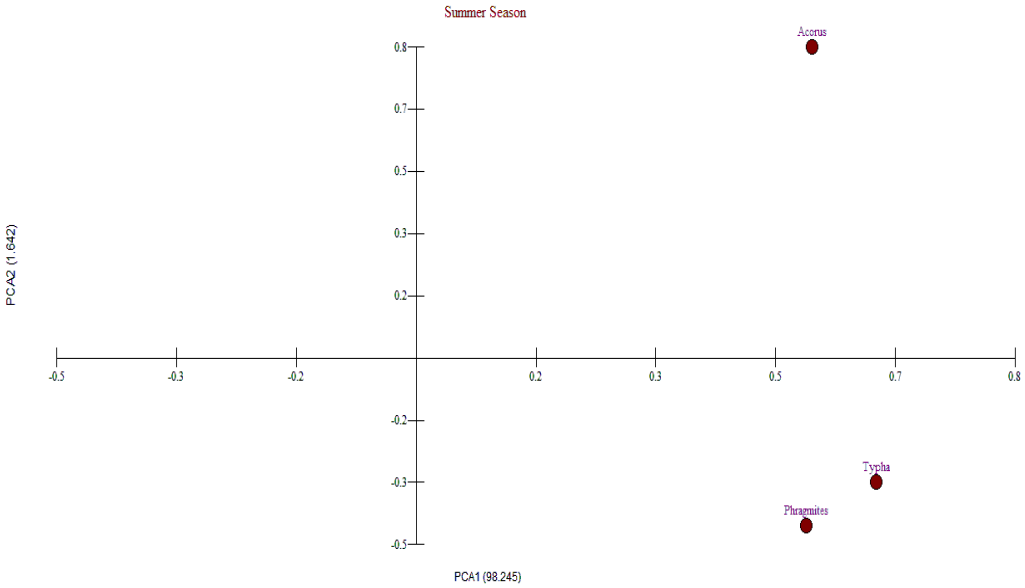

Figure 5: Principle Component Analysis (PCA) of root epiphytic bacteria of *A.calamus*, *T.latifolia*, *P.karka* during summer season based on phylum level.

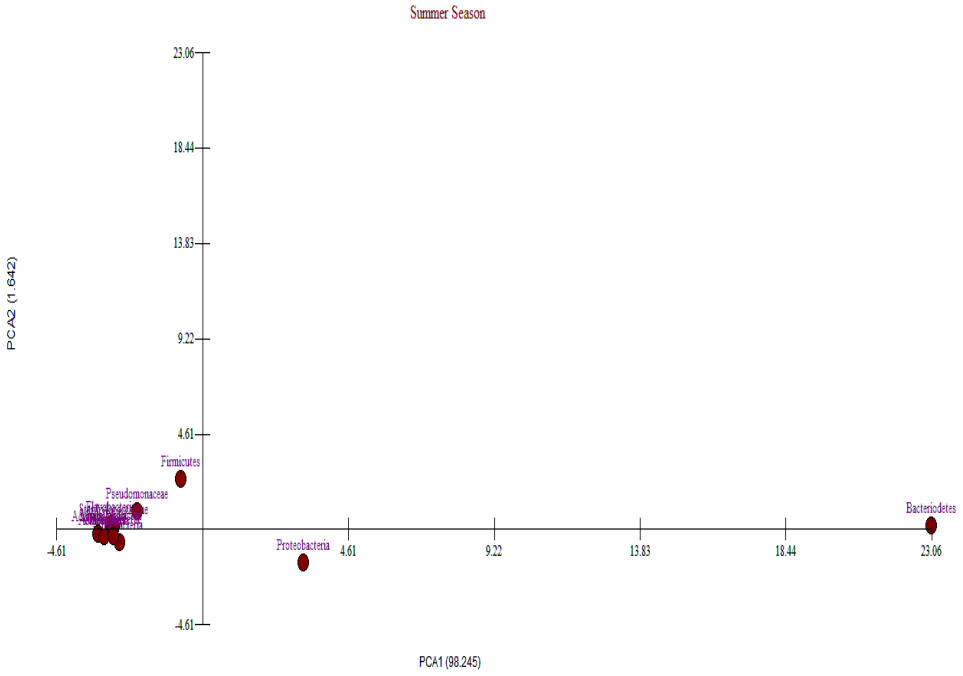

Figure 6: Principle Component Analysis (PCA) of root epiphytic bacteria during Summer Season based on species level.

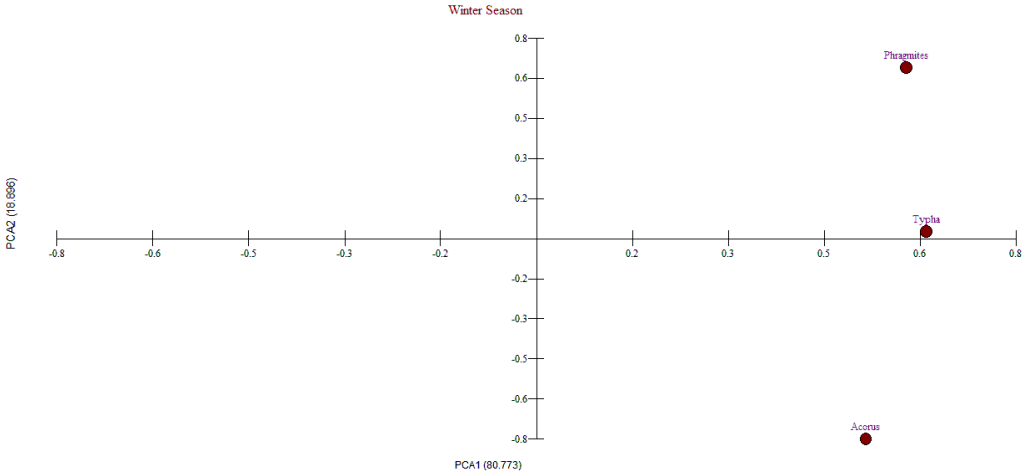

Figure 7: Principle Component Analysis (PCA) of root epiphytic bacteria of *A. calamus*, *T. latifolia*, *P. karka* during winter season based on phylum level.

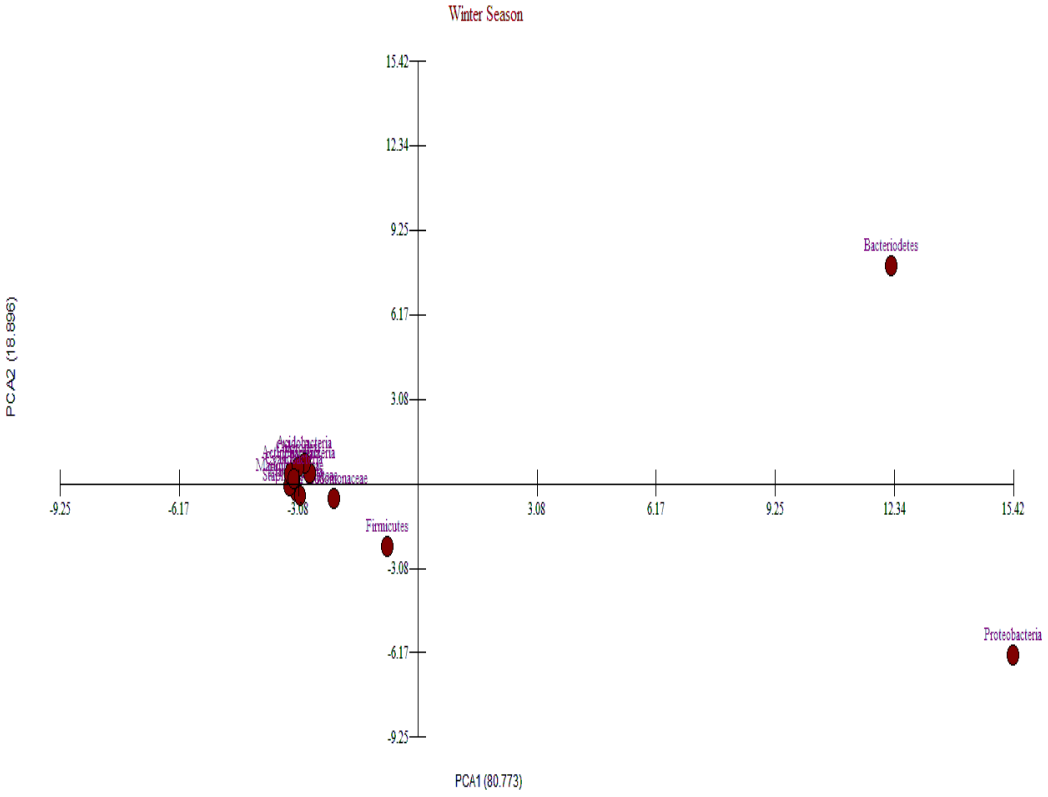

Figure 8: Principle Component Analysis (PCA) of root epiphytic bacteria of *A.calamus, T.latifolia, P.karka* during winter season based on species level.

Rarefaction curve was constructed during the winter season (Fig. 9, 10) it can be easily interpreted from the rarefaction curve that the *P. karka* had the highest diversity followed by *A. calamus* and *T. latifolia* during both the seasons.

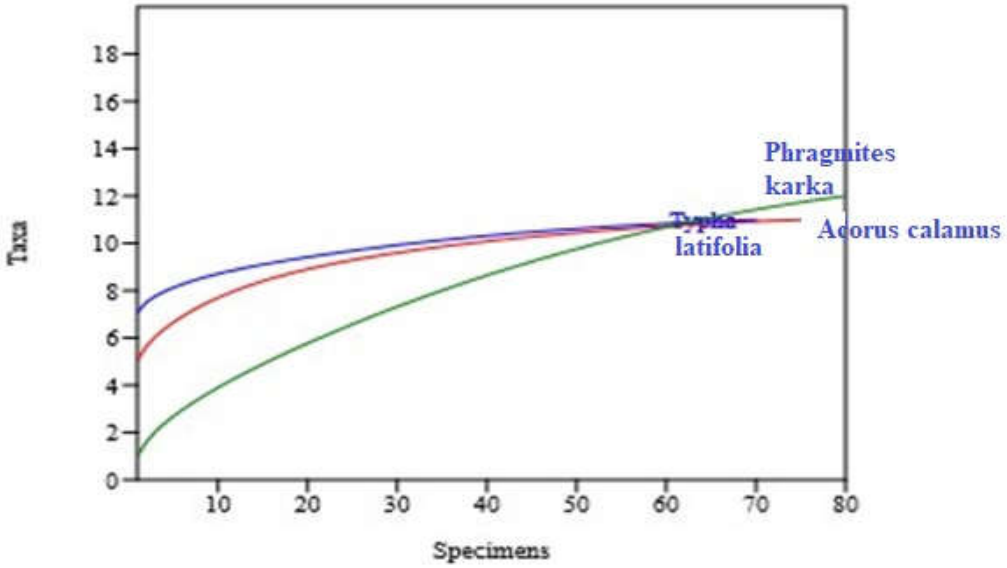

Figure 9: Rarefaction curve of the OTU's present in the three weedy plants during summer season; *A. calamus*, *T. latifolia* and *P. karka*.

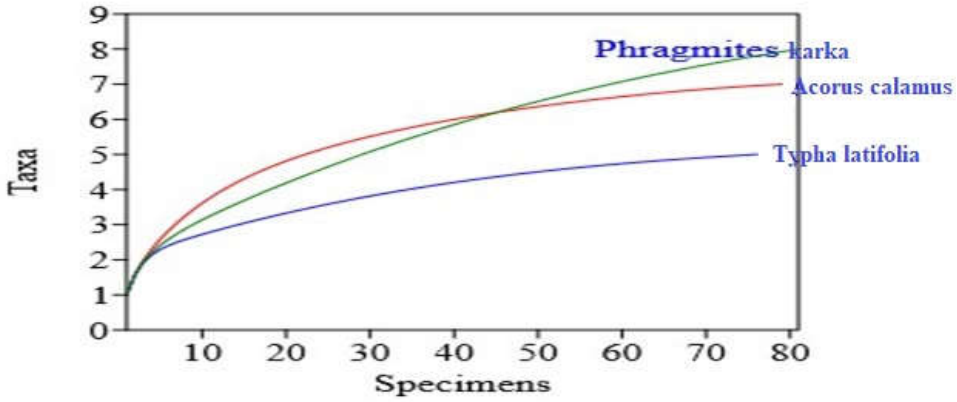

Figure 10: Rarefaction curve of the OTU's present in the three weedy plants during winter season; *A. calamus, T. latifolia, P. karka.*

Diversity indices of the bacterial community showed variation in all the three plants (Table 1). Shannon – Weiner diversity index (H′) which is used to characterize the diversity of community was evaluated for each sample. The value of H′ varied within the samples.

During the summer season the highest diversity index was observed for *P. karka* (H′ = 1.69), followed by *A. calamus* (1.256) and lowest diversity was reported in case of *T. latifolia* (H′ = 1.516). Whereas in the winter season the highest diversity index was observed for *P. karka* (H′ = 1.68) followed by *A. calamus* (1.516) and the lowest diversity index was reported in *T. latifolia* (H′ = 1.027) (Table 1).

DISCUSSION

Plants are in a complex relationship with bacterial communities and their interaction helps in supporting the expansion of the microbial population. Plants integrate the epiphytic bacteria in their defence mechanism in order to survive under stressed conditions. Thus, plant epiphytes play an important part in the plants defence mechanism against the environmental stresses[17].

In this present study, root epiphytic bacterial diversity analysis of wetland plants *A. calamus, T. latifolia* and *P. karka* was done. Fragsort analysis of the T-RFs thus obtained, with different sizes and relative percentage abundance helped us achieve the list of microorganisms. Thus providing the comprehensible description of different bacterial groups present in three wetland plants during both the seasons. The dominant epiphytic phyla among all the three plants during the summer season (Fig. 1) were found to be the members of phyla *Bacteriodetes, Proteobacteria* and *Firmicutes*. Whereas phylum *Actinobacteria* and *Acidobacteria* were reported in both *A. calamus* and *P. arka* but were absent in *T. latifolia*. On the basis of Shannon – Weiner diversity index (H′) which is used to characterize the diversity of the community the highest diversity index was observed for *P.karka*, followed by *A. calamus* and lowest was reported in case of *T. latifolia*.

Among the three plants during the winter season (Fig. 2), phylum *Proteobacteria*, followed by the members of the phylum *Firmicutes* and *Bacteriodetes* were observed. Phylum *Actinobacteria* and *Acidobacteria* were seen to be present in both *A. calamus* and *P. karka* but were absent in *T. latifolia*. Whereas phylum *Thermotogae* and *Defferibacteres* were observed only in *P. karka* among all the three plants. This result can also be correlated to the diversity indices (Table 1) observed during winter season on the basis of Shannon- Weiner diversity index (H′). There was significant variation in the relative abundance of the root microbial community in both the seasons [20, 21].

Rarefaction curve was constructed on the basis of a number of OTUs present demonstrated higher species richness in *P. karka* followed by *A. calamus* and *T. latifolia* during both the seasons. During the summer season (Fig. 9), the plateaus of *T. latifolia* and *A. calamus* merged later demonstrated the decrease in variation in the number of species but *P. karka* showed a different plateau altogether showed its species richness. Whereas in winter season (Fig. 10) all the three plants showed independent plateaus thus depicting the increase in variation of species richness.

Jaccard similarity index was used to demonstrate the difference in composition as well as the abundance of microbial community during both the seasons. During the summer season (Fig. 3), the coefficient of similarity observed among the bacterial phylum thus obtained was 0.675%, whereas phylum *Chloroflexi* showed a similarity index of 0.410%; the reason that phylum *Chloroflexi* showed less similarity is due to its low relative species abundance, as it is only present in *P. karka*. Phylum *Proteobacteria*, *Firmicutes*, *Bacteriodetes* and *Cyanobacteria* were present in all the three plants thus were grouped together, but phylum *Fusobacteria*, *Actinobacteria* and *Acidobacteria* were grouped together as they were present in *P.karka* and *A.calamus*. Whereas during the winter season (Fig. 4) it was observed that phylum *Deferribacteres* and *Thermotogae* showed a 0.375% coefficient of similarity and rest of the bacterial phyla showed a coefficient of similarity of about 0.675%. phylum *Deferribacteres* and *Thermotogae* showed less similarity owing to its low relative species abundance as these bacterial groups were reported only in *P. karka*. Phylum *Acidobacteria* and *Actinobacteria* were grouped together as they were present in both *P. karka* and *A. calamus*; whereas phylum *Chloroflexi*, *Cyanobacteria*,

Proteobacteria, Firmicutes, Bacteriodetes and *Fusobacteria* were all grouped together owing to their presence in all the three wetland plants [21,22].

PCA analysis demonstrated that the rhizospheric microbial diversity in all the three wetland plants differs in their properties as well as nutrient composition thus harbours a diverse microbial community. The reason behind diverse microbial community structure might be the presence of household waste as well as heavy metal contamination in the sewage water of IARI, thus bacteria capable of remediating or capable of surviving extreme toxicity can only persist in these conditions. Thus the presence of dominant *Proteobacteria* followed by *Firmicutes* and *Bacteriodetes* group during winters (Fig. 7 and 8) and a dominant bacterial group of *Bacteriodetes, Proteobacteria* and *Firmicutes* during summer (Fig. 5 and 6) can be attributed to the root exudates, contamination and presence of toxicities in the rhizospheric zone.

CONCLUSION

This study gives us an insight about the variable microbial community pattern in different season in the wetland plants; *Acorus calamus, Typha latifolia* and *Phragmites karka*. The microbial diversity varies in both the seasons in the epiphytic zones of these wetland plants as well as they vary in their physicochemical parameters and heavy metal concentrations. These epiphytic zones are dominated by phylum *Proteobacteria, Bacteriodetes* and *Firmicutes*. They also harbour specific bacterial diversity belonging to phylum *Deferribacteres* and *Thermotogae* were only present during the winter season in *P. karka*. Phylogenetic analysis and statistical analysis suggests the variation in the presence of species richness and evenness in the three wetland plants. Thus indicating the strong effect of the environmental factors along with physicochemical parameters and presence of contaminants such as heavy metal on the epiphytic microbial community of these wetland plants.

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CONFLICT OF INTEREST

We do not have any financial/commercial conflicts of interest.

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