DEPARTMENT OF BIOTECHNOLOGY, CUSAT

Impact of Flood/Landslides 2K18 on Biodiversity

Metagenomic analysis of soil microbial diversity in post flood mangroves

Dr. Sarita G Bhat

7/20/2019

Principal Investigator

Dr.Bindiya. E.S

Co- Principal Investigator

Submitted to

Kerala State Biodiversity Board

l. Introduction

Surveys after the Kerala flood 2018 have indicated that the nature of soil in the affected area has drastically changed, which in turn would alter the microbial constitution of the soil. Flooding or submerging an air-dry soil in water sets in motion a series of physical, chemical and biological processes that profoundly influence the quality of soil. Some of the impacts are restriction of gas exchange, depletion of molecular oxygen, accumulation of soil gases, changes in soil temperature, swelling of colloids, to name a few. The bacteria in the flood water are aerobic and the fauna largely zooplanktons. Along with the chemical changes in flooded lands, the microbial changes are also prominent. Aerobic bacteria along with algae and photosynthetic bacteria are present. In addition methane- oxidizing bacteria oxidize methane moving into the surface layer from the anaerobic layer. More changes in the microbial composition were reported by Gerard et al. 1984.

Mangrove ecosystems are unique ecological niches at the interphase between the marine and terrestrial environment that maintain genetically diverse groups of aquatic and terrestrial organisms. It occupies a diversified habitat like core forests, litter-forest floors, mud flats, water bodies (rivers, bays, creeks, etc.), coral reefs and sea grass ecosystems. A total of 4,011 species including 920 plants (23%) and 3,091 animals (77%) species have been recorded from Indian mangrove ecosystems, which is highest in the world. Among flora, marine algal diversity was greater with highest number of species (557 species) i.e. 60.1% followed by fungi 11.2%, mangrove associates 9.3%, bacteria 7.5% and mangroves 4.2% among others. Mangrove sediments are generally nutrient rich with a multiplicity of microbes playing important roles in nutrient recycling and various other ecological processes. Natural calamities are therefore known to impact the natural balance of the mangroves and disturb the fragile balance of this ecosystem.

The key step in metagenomic study is to choose an appropriate method for community DNA extraction from the environmental samples. Isolation of total community DNA itself is a challenge. This has been overcome and published to a certain extent (Nair et al., 2014). The study envisages the metagenomic analysis of the flood affected mangroves and unaffected Mangalavanam. The study also aims at a comparative study of post and pre flood impacts on soil microbiota. The data of pre flood metagenomic

analysis of Mangalavanam is available in the host institution. Since the whole metagenome is analyzed, the gene expressions can also be monitored other than biodiversity.

The complexity of the mangrove microbial communities has generated deep interest among microbial ecologists. The dynamic environment of the mangrove ecosystem, brought about by the regular tidal variations, pH, temperature, salinity, light, rainfall and nutrient availability provides an excellent environment for a wide range of organisms with diversified functional roles. Studies have shown that microbial communities play a vital role in this ecosystem, being essential for biogeochemical cycles and biocycling of most nutrients, including nitrogen. Metagenomic analysis of the Mangalavanam mangrove was studied earlier in the department where the work is proposed to be carried out (Nair et al., 2014). Imchen et al 2017carried out the first detailed analysis of microbial communities within the mangrove ecosystems from four different locations in mangrove forests across Kerala, India, and compared them to a similar Brazilian ecosystem, a tropical rainforest, and samples taken from ocean sediment.

2. Objectives

1. Collection of soil samples from mangroves.
2. DNA isolation from soil samples
3. Metagenomic analysis by NGS.

3. Materials and Methods

3.1 Study Sites (District, Panchayat with Map, Area, Landscape, Ecosystems, Type of Disaster as flood, landslides) (Table 1).

Table 1: Sampling sites including Latitude and Longitude

Latitude (N)	**Latitude (E)**	**Location of Survey**	**Type of data/product**	Type of Disaster
09° 58′	76° 14′	Puthuvypin in Ernakulam district	Collected Soil from 5 locations	Flood

09° 59′	76° 16′	Mangalvanam Birds Sanctuary of Ernakulam district	Collected Soil from 5 locations	Flood
10° 065′	**76° 24′**	**Kadamakkkudy of Ernakulam District**	**Collected Soil from 5 locations**	**Flood**
11° 23′	75° 79′	Kallai river bank of Kozhikode	Collected Soil from 5 locations	Unaffected
11° 13′	75° 82′	Kadalundi river bank in border of Malapuram and Kozhikode.	Collected Soil from 5 locations	Unaffected

3.2 Methodology

- BMC Meeting/ FDGs/ Stakeholder Consultations etc

The region Kadamakkudy was not initially included in the proposal. This sampling site was included in the study due to the recommendation of BMC co-ordinator of Ernakulam district. Kadamakkudy region was immensely flooded during Kerala flood 2018 (Table 1).

- Survey Methodology Including Period of Survey **(**Collection of soil samples from mangroves)

The samples were collected from one feet depth using a sterile spatula and transferred to pre-autoclaved sterile glass bottles with rubber stoppers. The sampling was done in the post monsoon period (February – March) 2019. The samples were brought to the laboratory and stored under refrigeration temperature.DNA isolation using MoBioUltraCleanTM soil DNA isolation kit, USA (Commercial DNA extraction kits are now commonly used for extraction of high molecular weight DNA from complex habitats. Studies evaluating various commercial kits to other methods have shown that DNA yield and purity vary based on methodology and soil type).

- **Whole metagenomic sequencing**

Nucleic acid quality and quantity were checked via Nanodrop 2000 where after 1µg of DNA was used to prepare sequencing libraries. Sequencing libraries were prepared for R7.3 flow cells run on an original MinION device using the Genomic DNA Sequencing Kit SQK–MAP005 (version 5 chemistry) according to the base protocol from Oxford Nanopore with slight modifications (Ip et al., 2015) and for flow cells run using the Nanopore Sequencing Kit SQK– MAP006 (version 6 chemistry) according to the manufacturer's recommendations. The steps for library SQK–MAP005 preparation included in this order: shearing 1µg in a Covaris g-TUBE (Covaris, Inc., Woburn, MA, USA) at 2000 × g for 2 min, treatment with PreCR (New England Biolabs, Beverly, MA, USA), cleanup with 1× AMPure beads (Agencourt, Beckman Coulter, Brea CA, USA), end-repair with NEBNext End Repair Module (New England Biolabs), cleanup with 0.5× AMPure beads, dA-tailing with NEBNextdA-Tailing Module (New England Biolabs), ligation to a cocktail of both the leader and hairpin sequencing adapters (Oxford Nanopore Technologies) using Blunt TA Ligase (New England Biolabs), clean up using his-tag Dynabeads (Life Technologies, Carlsbad, CA, USA), and recovery of the presequencing mix in 25µL of Elution Buffer (Oxford Nanopore Technologies). After priming the flow cell with EP solution according to the manufacturer's recommendations, an initial 6-µL aliquot of the presequencing mix (at 10–20 ng/µL) was combined with 141 µL EP Solution and 3 µL Fuel Mix and applied to the flow cell. Thereafter, at 6- to 8-h intervals, additional presequencing mix aliquots (held on ice) combined with EP Solution and Fuel Mix were added to the flow cell at times roughly coinciding with reprogrammed pore "remux," which is a process that adjusts the bias voltage and mux channels to maximize yield performance. Modified scripts (J. Tyson, personal communication) caused the MinION device to perform four remux steps at 8-h intervals to maintain regular increases in data.

Steps for library SQK–MAP006 preparation included in this order: shearing in a Covaris g-TUBE (Covaris, Inc.) at 2000 × g for 2 min, treatment with PreCR (New England Biolabs), cleanup with 1× AMPure beads (Agencourt, Beckman Coulter), combined end-repair and dA-tailing with NEBNextUltraII End Repair/dATailing Module (New England Biolabs), cleanup with 1× AMPure beads, ligation to a cocktail of both the leader and hairpin sequencing adapters (Oxford Nanopore Technologies) using Blunt TA Ligase (New England Biolabs), addition of a tether to the hairpin

segment, cleanup using MyOne Streptavidin C1 Beads (LifeTechnologies), and recovery of the presequencing mix in 25µL of Elution Buffer (Oxford Nanopore Technologies). After priming the flow cell with running buffer and fuel according to the manufacturer's recommendations, an initial 6-µL aliquot of the presequencing mix (at 10–20 ng/µL) was combined with 75µL Running Buffer, 65µL water, and 4µL Fuel Mix and applied to the flow cell. Thereafter, at 8-h intervals, additional presequencing mix aliquots (held on ice) were combined with Running Buffer and Fuel Mix and added to the flow cell at times roughly coinciding with reprogrammed pore remux(modified scripts from J. Tyson, personal communication). Modified remux scripts were not used for the final MinION run (staggered community analysis), because that run was controlled by a new version of MinKNOW.

- **Sequence comparison and analysis**

Sequencing of whole genome libraries can enhance environmental metagenomic analysis by providing more precise identification of the composition and structure of the community than is possible by amplicon sequencing of marker genes (e.g.,16S). Typical environmental samples contain tens of thousands to millions of organisms, yet the resulting metagenomes almost certainly under represent this diversity and, often due to short-read strategy, the resulting data sets can be confidently assigned only to higher taxonomic levels. The bioinformatic methods used in this analysis was One Codex which comparesthe input reads against their own more concise reference databases, providing an assignment for the most likely origin of each individual sequence.

4. Results (As applicable according to objectives)

4.1. Assessment of microbial diversity of selected areas

The soil samples were collected from Mangalavanam, Puthuvypin, Kadamakkudy, Kallai and Kadalundi. The samples were dried ground well and used for DNA isolation. The sampling sites are shown in figure (Fig. 1, Fig. 2 Fig. 3, Fig. 4and Fig. 5).

4.1.1.DNA isolation from soil samples

DNA isolation was done from 5g of soil from all the sites.

Table 2: Concentrations of metagenomic DNA isolated from different locations of the study

Location 1	**Mangalavanam Samples**	**Conc of DNA (ng/mL)**
	MV1	229.3
	MV2	132.6
	MV3	35.6
	MV4	92.8
	MV5	57.9
Location 2	**Puthuvypin Samples**	**Conc of DNA (ng/mL)**
	PV1	13.4
	PV2	30.3
	PV3	7.8
Location 3	**Kadamakkudy Samples**	**Conc of DNA (ng/mL)**
	KD1	42.7
	KD2	78.1
	KD3	16.1
	KD4	25.9
Location 4	**Kallai**	**Conc of DNA (ng/mL)**
	KAL1	240.6
	KAL2	225.4
	KAL3	188.2
	KAL4	280.9
	KAL5	190.4
Location 5	**Kadalundi**	**Conc of DNA (ng/mL)**
	KDL1	154.3
	KDL2	167.3
	KDL3	120.7

The isolated DNA was run on agarose gel electrophoresis (Fig 6) and found that the samples are of good quality. The concentrations of metagenomic DNA obtained were checked on NanoDrop 2000 (Table 2). 280/260 and 280/230 ratios were good for sequencing. DNA obtained from Puthuvypin region was less compared to all other locations. This may be due to the reduced microbial diversity present in the soil.

4.2. Assessment of Microbial Biodiversity of Selected Areas

In Kadamakkudy samples **93**% of 9,616 the sequences obtained were unclassified. In Mangalavanam sample **93.57**% of 917 reads were unclassified sequences where as **21.88**% of 32 reads were readable in Puthuvypin sample (Table 3). The diversity distribution including Shannon and Simpson indices are shown in fig. 7 and 8.

Table 3: Details of readable sequences from the metagenomic data

Sample	**Number of reads**	**% Classified Reads**
Mangalavanam	917	6.43
Puthuvypin	32	21.88
Kadamakkudy	9616	7
Kadalundi	16,214	14.31
Kallai	15,269	4.49

4.2.1 Phylum level distribution

In all the locations Proteobateria was the prominent phylum. In Mangalavanam location, only nine phyla were noted. The prominent phylum being Proteobacteria followed by Bacteriodetes (Fig. 9A). The least number of phyla were identified at Puthuvypin region. The present analysis could identify only three phyla, with Proteobacteria dominating. Fig 9B). Twenty one phyla were identified in the Kadamakkudy region other than the uncultured phyla. The most prominent phylum was Proteobacteria followed by Actinobacteria (Fig. 9C).

4.2.2. Species level distribution

Species level distribution and phylogenetic tree was plotted using One Codex web portal. The phylogenetic trees obtained could clearly depict the species diversity of the mangrove regions as visualized in the figures (Fig. 10, 11 12, 13 and 14). We can understand that the species diversity is more in Mangalavanam mangrove and less in Puthuvypin mangrove.

4.2.3. Microbial Species Affected By Flood

Flooding of the soil had a strong effect on the bacterial community structure. Flooding decreased the relative sequence abundance of the Acidobacteria, Chlorobi and Proteobacteria (Table 4).The decrease in Proteobacteria is not huge in Mangalavanam as cited/recorded in other flood related studies (de León-Lorenzana et al., 2017). This may be because of the increase in certain families such as Pseudomonadaceae, Xanthomonadaceae and Rhizobiales in Mangalavanam whereas the families like Vibrionaceae and Halomonadaceae were missing from Managalavanam. In Puthuvypin mangrove, only Rhizaobale family from Proteobacteria was present, whereas in Kadamakkudy mangrove there was an appalling abundance in Vibrionaceae family (Fig. 15). Vibrionaceae family was not found in Kallai and Kadalundi mangroves. Thus the phylum diversity of Kallai and Kadalundi mangroves are mostly related to pre flood data of Mangalavanam Mangroves. The relative abundance of most other bacterial phyla, i.e., Actinobacteria, Bacteroidetes, Planctomycetes and the unassigned phylotypes showed an opposite pattern and increased after the first flooding (Fig. 16).

Table 4: Distribution of Selected microbial phyla in post flood and pre flood scenario

Phylum	**Relative Abundance (%)**					
	MV (PRF)	**MV (POF)**	**PV**	**KD**	**KAL**	**KDL**
Proteobacteria	65	58	19	67	67	66
Acidobacteria	2	0	0	1	1	4
Chlorobi	1	0	0	0	0	0

4.4. Impact on Ecosystem

4.4.1. Immediate impact of disaster as top soil loss, sand piping etc

The overall effect of flood on soil communities when compared to the pre-flood data is that the total reads and amount of DNA was less in the flood affected samples. The Kallai and Kadalundi samples contain bulk reads in congruence to the pre flood data of Mangalavanam. The emergence of novel microbial families Ignavibacteriae (Iino et al., 2010), in Mangalavanam, Kadalundi and Kallai mangroves and Calditrichaeota (Marshall et al., 2017), Mucromycota (fungi) in Kadamakkudy mangrove was noticed.

4.4.2. Long term Modification in Ecosystems/ Microhabitats

Many microbial phyla which were prominent in our Pre flood analysis were missing in the post flood analysis. Acidobacteria, Chlorobi, Chloroflexi, Cyanobacteria, Euryarchaeota, Nitrospirae, Spirochaetes, Verrucomicrobia and so many were missing from Mangalavanam mangrove following the floods.

4.4.3. Impact on Biodiversity

The loss of microbial diversity of Puthuvypin is thought provoking. This will have a long term impact on the biodiversity of this area. The lack of a healthy soil and loss of tree canopy will hinder the natural processes to recoup the loss in this area.

5. Environmental Impact

Though the proposal did not include the chemical analysis of soil, we conducted an analysis to find out the amount of heavy metals in the soil. The heavy metal concentrations of all the survey areas were high. The reduced diversity of plants, increased shrimp culture and higher heavy metal concentration in Kadamakkudy mangrove (Fig 15) hints at biomagnification and its associated hazards in the environment. The conserved mangrove forests are superior in their diversity health than others.

6. Socio Economic Impact

The increased Vibrio population in Kadamakkudy region can be expected to have a high socio economic impact. Since this is an area of extensive aquaculture, there will be higher occurrence of Vibrio infections in the aquaculture farm leading to huge economic loss. The second possibility is that Kadamakkudy is a place with human dwelling. Thus there is a chance for outbreaks such as cholera, vibriosis and other Vibrio related diseases. Vibriosis causes an estimated 80,000 illnesses and 100 deaths in the United States every year (WHO, 2011). Control of cholera is a major problem in several Asian countries as well as in Africa.

7. Discussion & Conclusion

Yu et al., 2018 published a report on the impact of Hurricane Harvey on pathogenic indicator bacteria and resistance genes. de León-Lorenzana et al., (2017) published a manuscript on the study of flooding in soil which affected the soil microbial diversity. Our present study is also supported by the findings of de León-Lorenzana et al., (2017).

8. Suggested Interventions

8.1 Prioritized Areas (Short term, medium term and Long term)

Short term: Kallai mangrove – to conserve

Medium term: Mangalavanam - to reinstate microbial diversity

Kadamakkudy: To make the aquaculture ecofriendly, sustainable and productive; to grow more mangrove plants

Long term: Long period will be required for restoration of Puthuvypin mangroves and establish their related microbial niche.

8.2. Issues

- Prevalence of antibiotic resistance genes should be studied in Kadamakkudy region
- Microbiome of each mangrove plant in relation to microniche
- This will be helpful while introducing mangrove plants in areas like Puthuvypin

- Seasonal studies on the composition of soil microbes is needed (Pre monsoon and post monsoon) for few more years
- The real impact of the flood will only be apparent a few years later.
- Human interference in mangrove areas are a great threat
- Commercial constructions in the nearby areas of mangrove forests have a detrimental impact on the ecosystem and in long term will impact coastal habitations

8.3 Interventions suggested by BMC/ LSG: Nil

8.4 Suggested site specific restoration activities

Intensive restoration activities are recommended in Puthuvypin and Kadamakkudy mangroves. The restoration activity will be helpful even to reduce the high heavy metal ion concentration in Kadamakkudy mangrove. The height of the nearby structures of Mangalavanam mangrove should be monitored and controlled so that the migratory birds could fly to reach their destination before they perish by hitting on tall buildings.

8.4 Landscape level Restoration Activities: Nil

9. Ambiguities in the study

(Ambiguities can include short time period, Migratory sps, Breeding time etc)

Study of impact of flood is a long term process and the period used for the study was very short, even though this study could shed some light towards the importance of biodiversity and its conservation.

10. References

- Yu, P., Zaleski, A., Li, Q., He, Y., Mapili, K., Pruden, A., ... & Stadler, L. B. (2018). Elevated Levels of Pathogenic Indicator Bacteria and Antibiotic Resistance Genes after Hurricane Harvey's Flooding in Houston. *Environmental Science & Technology Letters*, *5*(8), 481-486.

- de León-Lorenzana, A. S., Delgado-Balbuena, L., Domínguez-Mendoza, C., Navarro-Noya, Y. E., Luna-Guido, M., & Dendooven, L. (2017). Reducing salinity by flooding an extremely alkaline and saline soil changes the bacterial community but its effect on the archaeal community is limited. *Frontiers in microbiology*, *8*, 466.
- Risk assessment of Vibrio parahaemolyticus in seafood, Interpretative Summary And Technical Report, World Health Organization Food And Agriculture Organization Of The United Nations 2011.

11. Contributors and Acknowledgements

Contributors:

1. Dr. Sarita G Bhat, Principal Investigator and Head, Department of Biotechnology, CUSAT
2. Dr. Bindiya. E.S, Co-Principal Investigator, KSBB Project, Department of Biotechnology, CUSAT.

Acknowledgement

We sincerely acknowledge Dr. Sreekanth P.M., Co-Principal Investigator, KSBB project for the help rendered in selecting the areas of priority for the study, the identification of mangrove plants and their peculiarities. His assistance during the discussions was noteworthy. We also thank Mr. Rinu Madhu P for kindly handing over the pre flood analysis data of Mangalavanam mangrove. We also thank Mr. Shaiju, Mr. Manu and Mr. Ayyappan for their assistance during sampling. We also acknowledge Cochin University of Science and Technology for providing the facilities needed for the study.

Plates

12. Plates

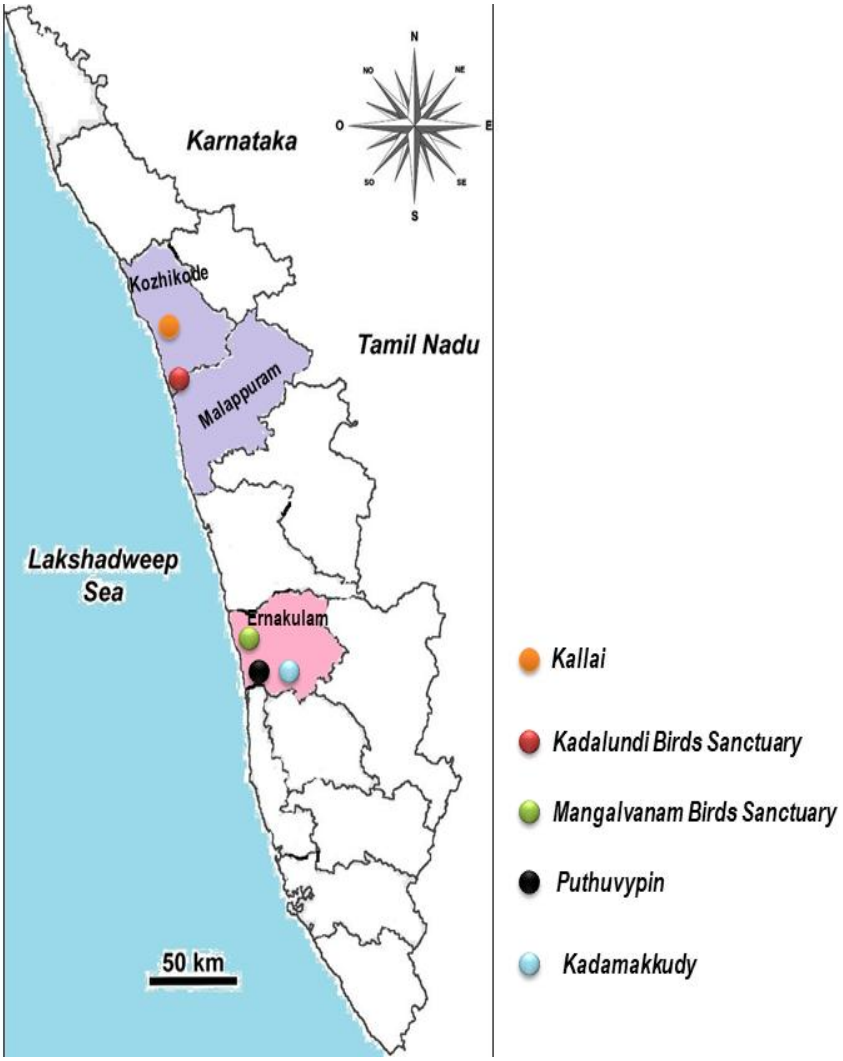

Fig: 1Mangalavanam mangrove sampling site

Fig: 2 Puthuvypin mangrove sampling site

Fig: 3 Kadamakkudy mangrove sampling site

Fig: 4 Kadalundi sampling site

Fig: 5 Kallai sampling site

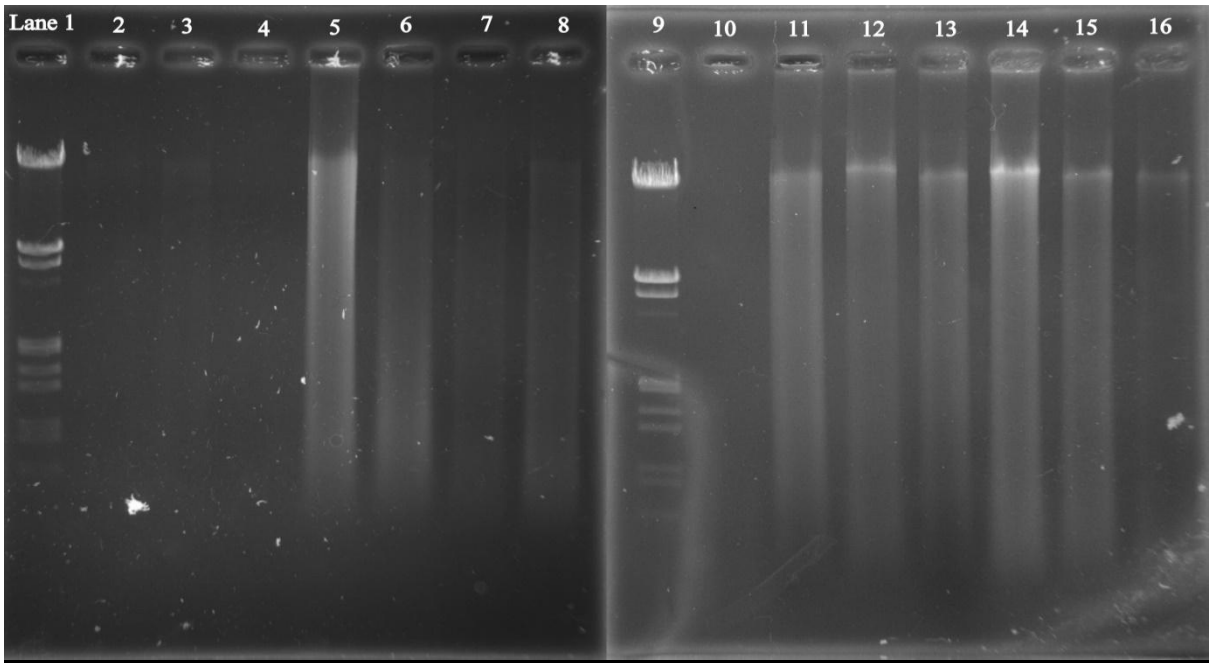

Fig: 6 Agarose gel electrophoresis of metagenomic DNA samples isolated from soil. Lane 1 and Lane 9 – Lambda DNA Hind 3/EcoR1 digest (Marker), Lane 2 to 4 – Metagenomic DNA from Puthuvypin sample, Lane 5 & 6 Metagenomic DNA from Mangalavanam sample, Lane 7 & 8 - Metagenomic DNA from Kadamakkudy sample, Lane 11 to 13 - Metagenomic DNA from Kallai sample, Lane 14 to 16 - Metagenomic DNA from Kadalundi sample.

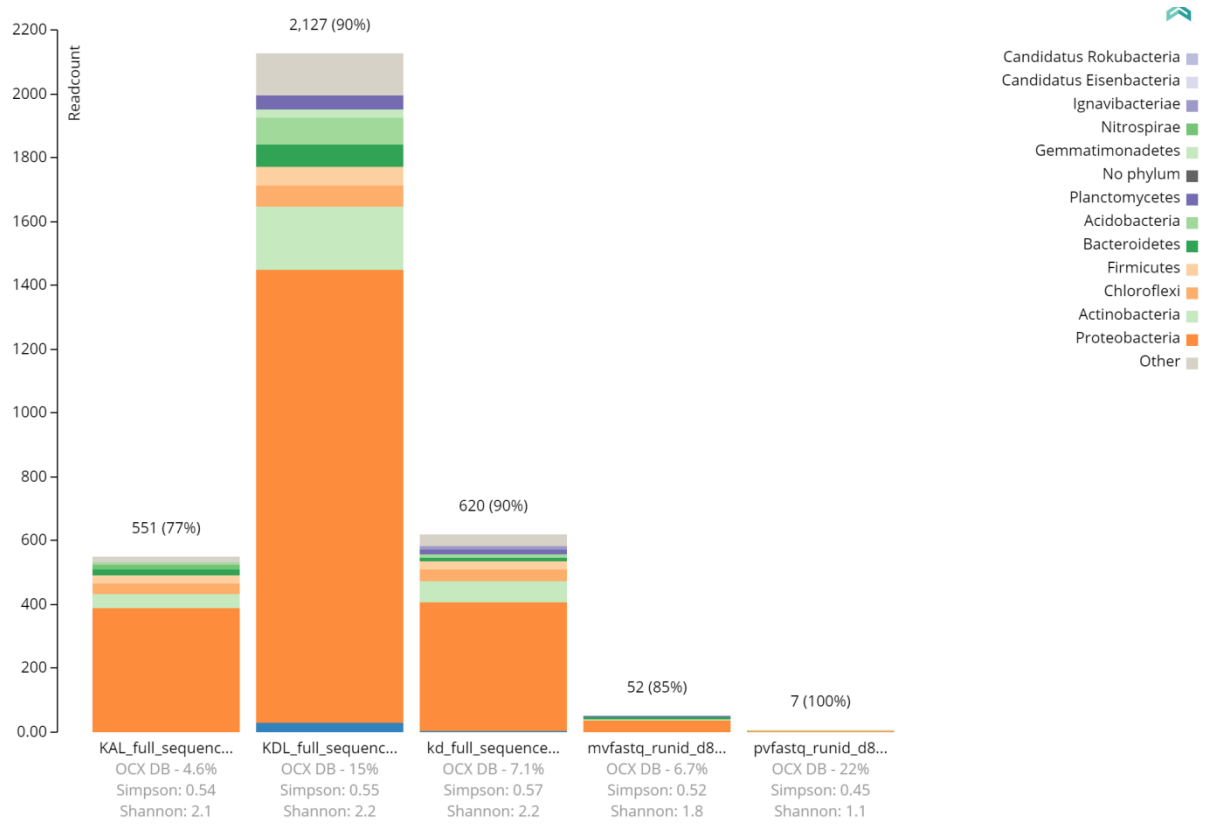

Fig:7 Comparison of diversity in the phylum level in all the sampling sites. The Simpson and Shannon indices are also shown.

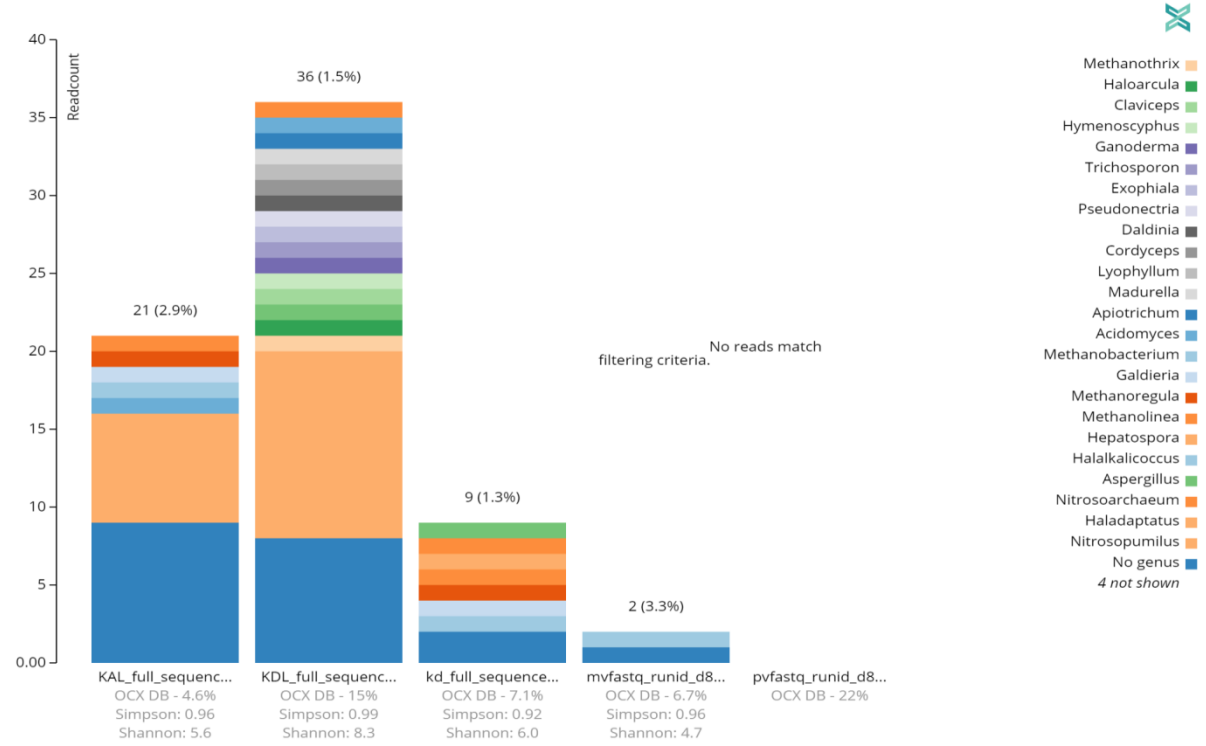

Fig:8 Comparison of diversity in the phylum level (Other than bacteria) in all the sampling sites. The Simpson and Shannon indices are also shown.

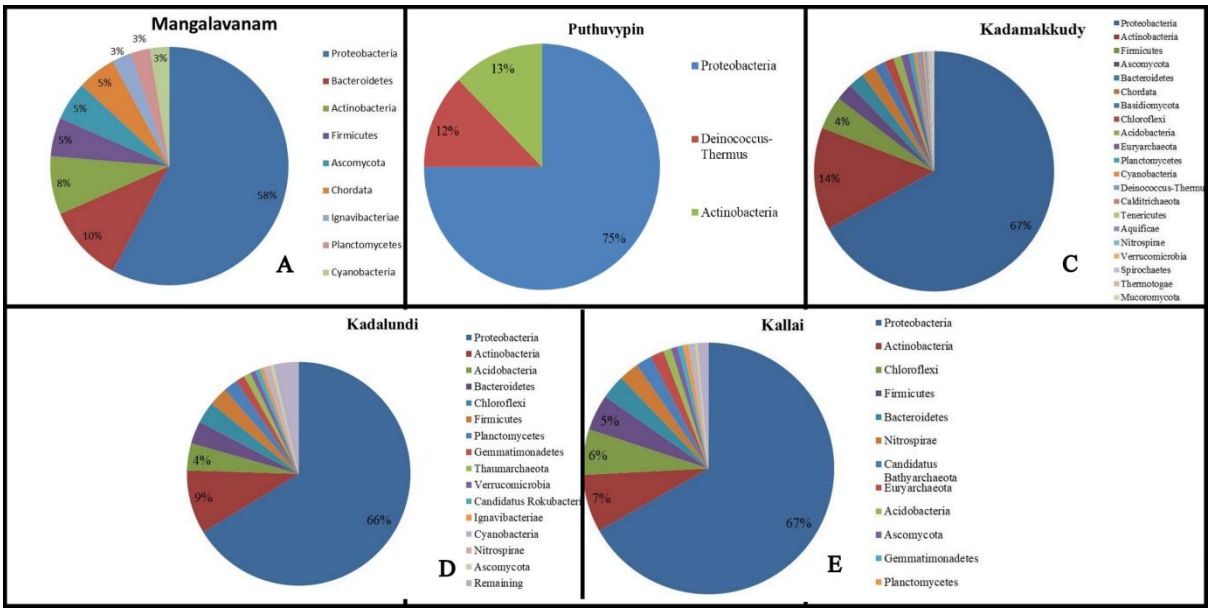

Fig:9 Comparison of phylum level distribution of A) Mangalavanam, B) Puthuvypin, C) Kadamakkudy, D) Kadalundi and E) Kallai mangroves

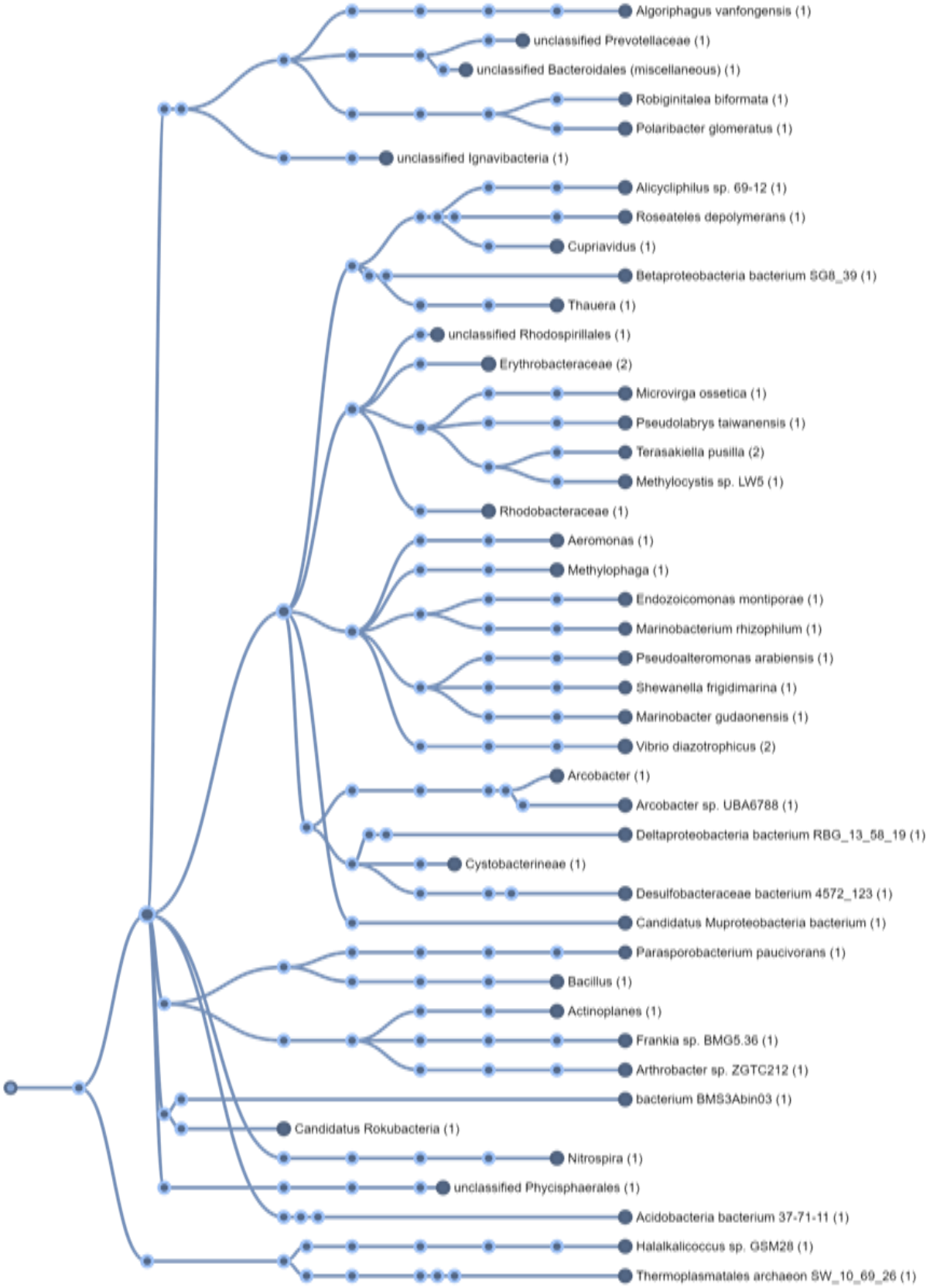

Fig.10 Species diversity of Mangalavanam mangrove

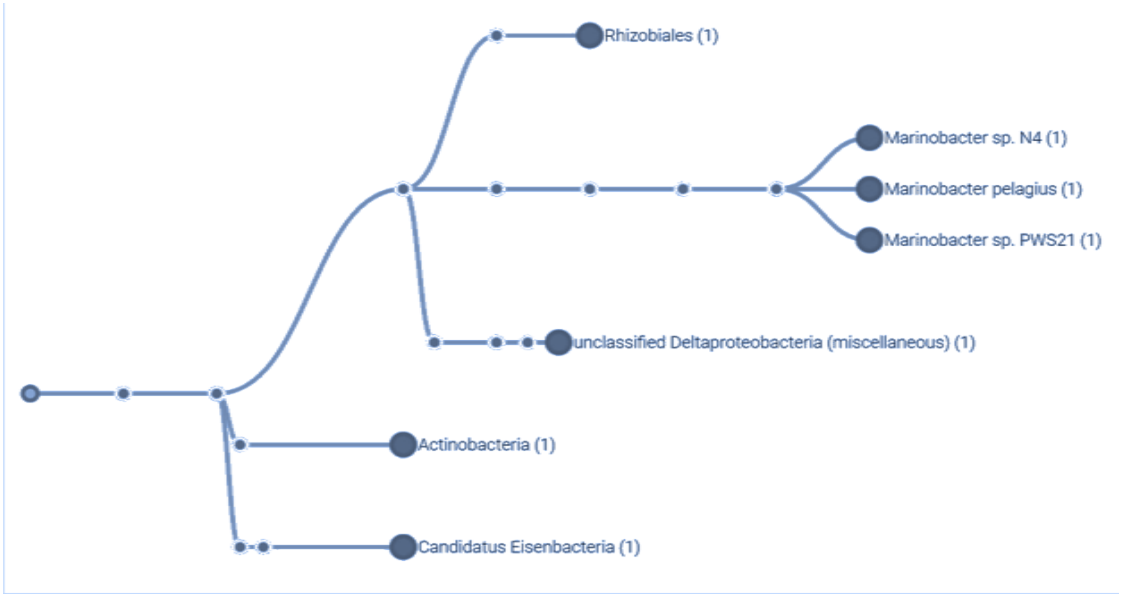

Fig.11 Species diversity of Puthuvypin mangrove

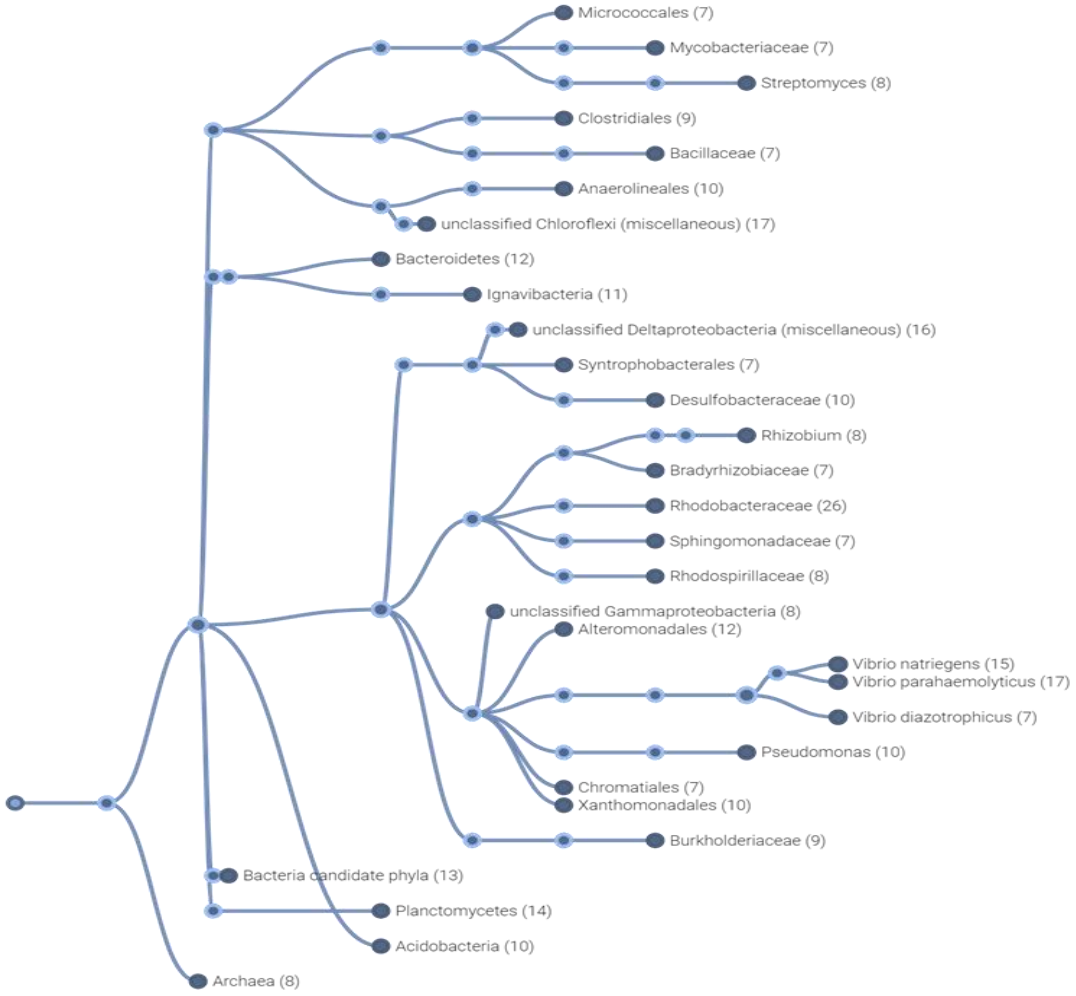

Fig.12 Species diversity of Kadamakkudy mangrove

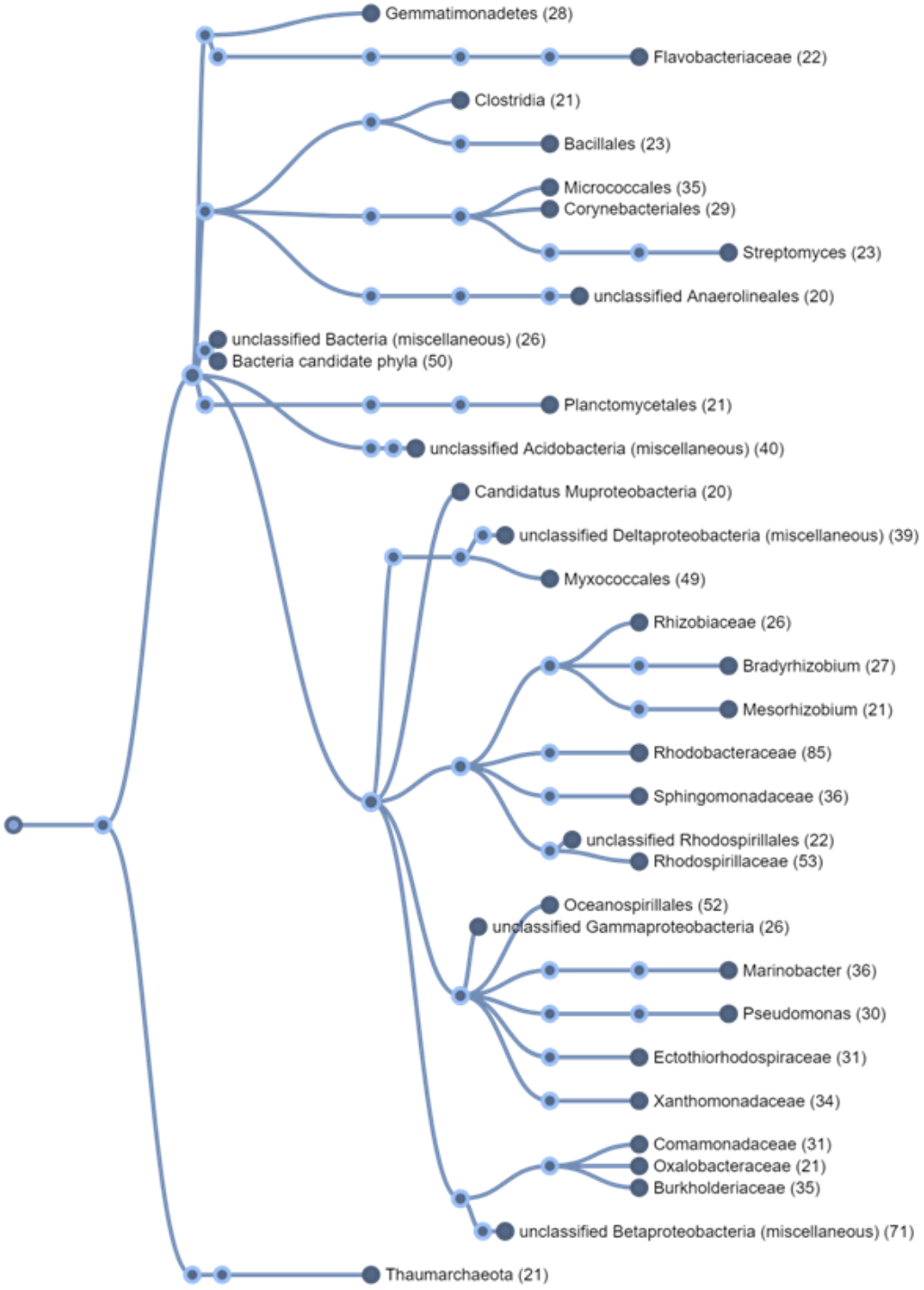

Fig.13 Species diversity of Kadalundi mangrove

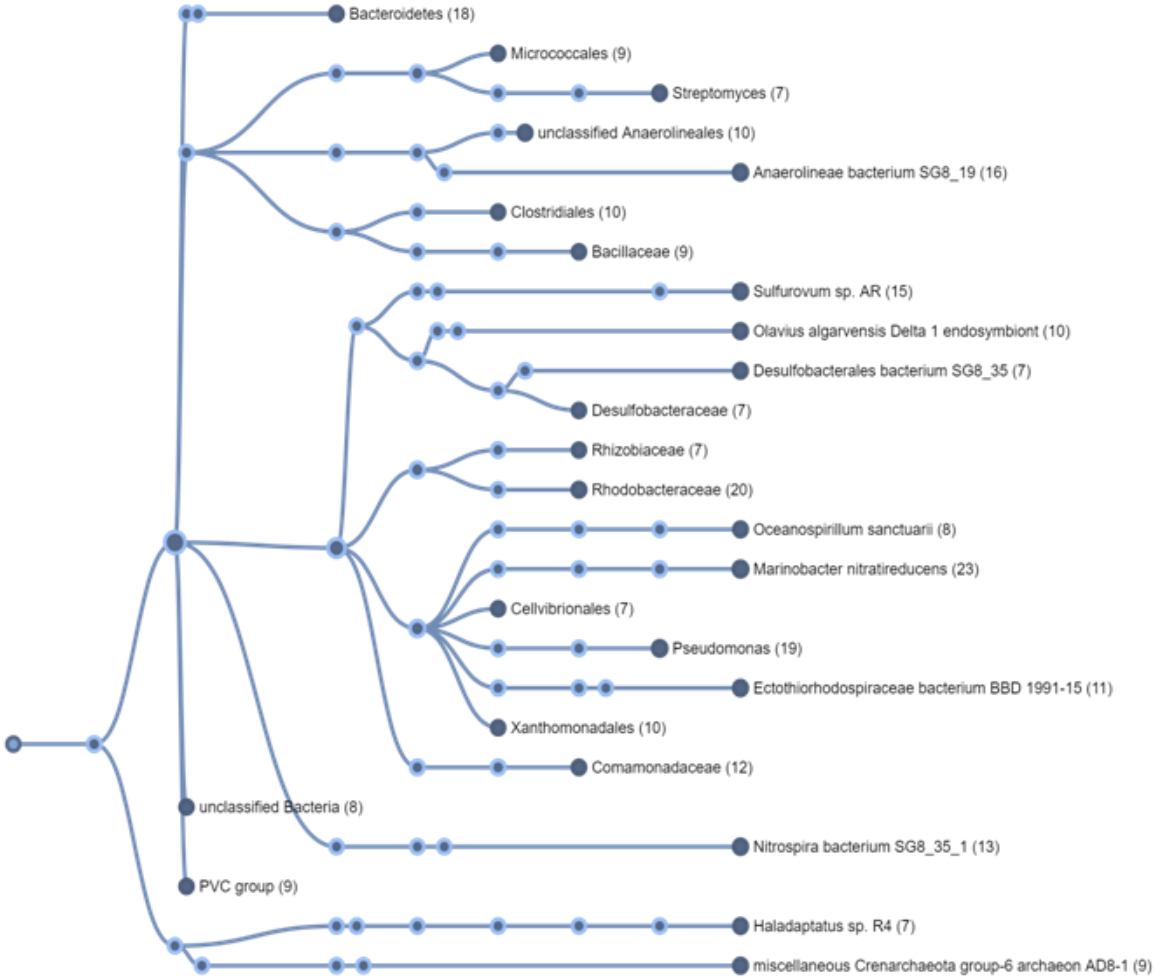

Fig.14 Species diversity of Kallai mangrove

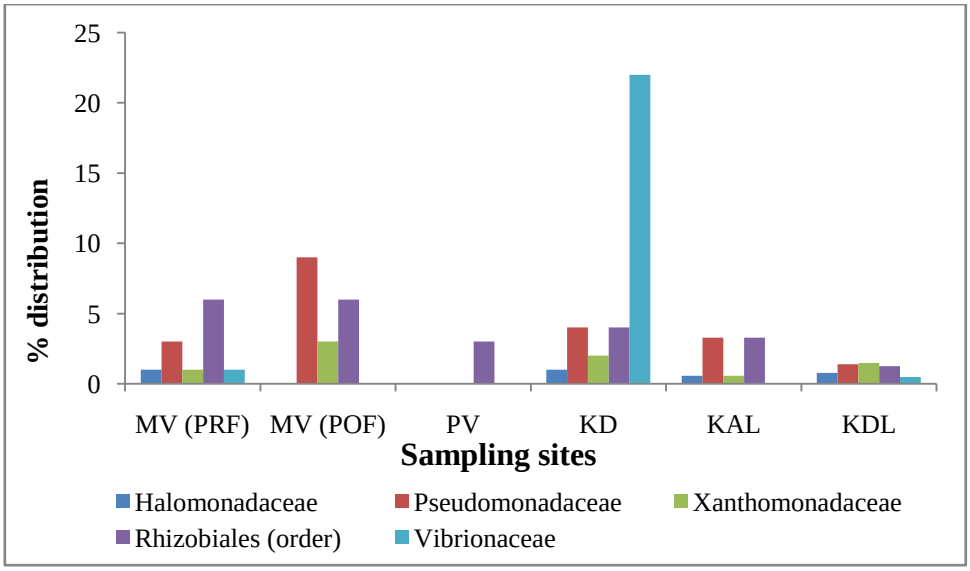

Fig 15. Distribution of Selected microbial family which come under Proteobacteria in post flood and pre flood scenario

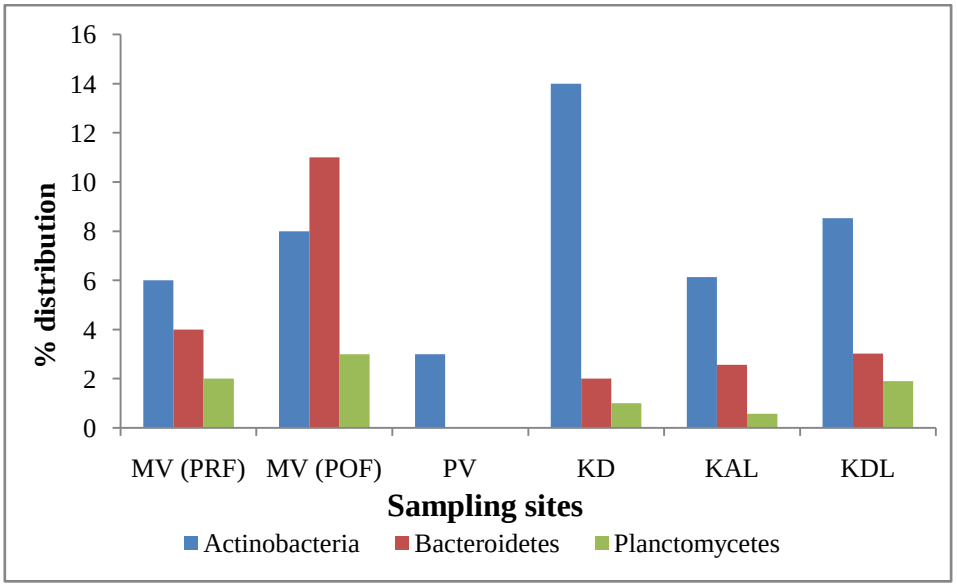

Fig. 16 Increase of certain phylum in post flood data.

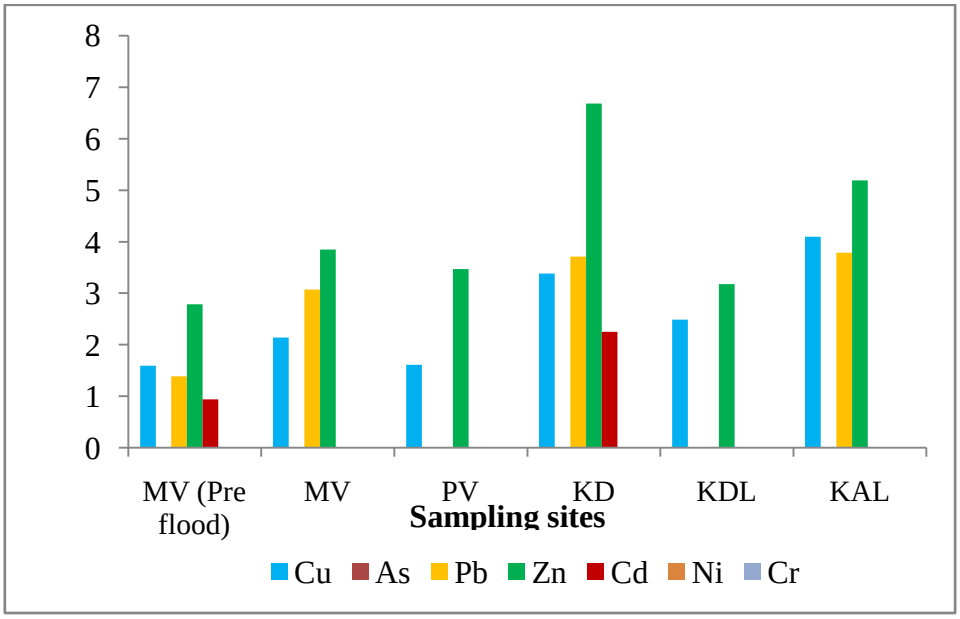

Fig. 17 Concentration of heavy metals in soil

13. fnd*r