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A study of microbial diversity and its interaction with nutrients in the sediments of Sundarban mangroves

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Mangroves provide a unique ecological environment for diverse microbial communities. They are particularly important in controlling the chemical environment of the ecosystem. Sundarban, being a rapidly changing ecosystem, is under stress due to various anthropogenic activities. The present study was taken with an objective to assess the microbial (fungal and bacterial) diversity with respect to behaviour of nutrients. Three sampling location viz. Canning, Jharkhali and Pakhiralay, were chosen based on anthropogenic stress. It was observed that at Canning, nitrate (7.46 mg.L^{-1}) and phosphate (8.12 mg. L^{-1}) in water were maximum of all the three locations. Total bacterial load (29.83 × 10^6), Phosphorus solubilising (14.08 × 10^4 CFU.g^{-1}), N_2 fixing (13.67 × 10^4 CFU.g^{-1}) and nitrifying bacteria (13.67 × 10^4 CFU.g^{-1}) as well as exchangeable phosphorus (42 µg.g^{-1}) was highest in the sediments collected at Canning. Sediments associated with dense mangroves (Pakhiralay) showed highest count of cellulose degrading bacteria (45.15 × 10^4 CFU.g^{-1}). Fungal diversity was also assessed and it was observed that *Aspergillus* and *Penicillium* were the most abundant species in the three sampling locations. The study had elucidated the existing environmental conditions played a significant role in the determination of microbial diversity as well as nutrient behaviour in the sediments.

Introduction

Mangroves are among the most productive coastal ecosystems in the world, confined to the tropics and subtropics and are estimated to cover an area of 1.7 to 2.0×10^5 km^2. Mangroves provide a unique ecological environment for diverse bacterial communities. These bacteria largely control carbon, phosphorus, nitrogen and sulphur dynamics and may contribute to soil and vegetation patterns.[1] Subsurface bacterial communities (along with epibenthic microalgae) may sequester nutrients and hold them within nutrient-limited mangrove[2]. N_2-fixing *Azotobacter*, potential bio-fertilizers, is abundant in the mangrove habitats of Pichavaram, South India.[3]

The mangroves are the second important habitat for marine fungi after driftwood. Fungi are vitally important to nutrient cycling in these habitats as they greatly facilitate the decomposition of mangrove material.[4] Hyde[5] listed 120 species from 29 mangrove forests around the world. These included 87 Ascomycetes, 31 Deuteromycetes and 2 Basidiomycetes. The samples collected from from Lakshadweep Island yielded 32 species[6] and 39 species of fungi were found in mangrove samples from the Maldives.[7] 48 fungal species were found in decomposing *Rhizophora* debris in Pichavaram, South India.[3] Frequency of occurrence of mangrove fungi along the east coast of India has been evaluated.[8] Sundarbans, the largest single chunk of prograding delta at the mouth of Ganges, is a unique bioclimatic zone in a typical geographical situation in the coastal region. Approximately, more than 50% of the north-western parts of deltaic and intertidal lands of Sundarbans delta have been continuously cleared for human habitation and settlement, agriculture and brackish water fisheries.[9] Various studies have been carried out in order to quantify the microbial load in Sundarban mangrove[10], however, there are few studies explaining the inter-relation of microbial diversity with nutrient behaviour. The present study was aimed to quantify the bacterial load (Cellulose degrading, N_2 fixing, nitrifying, and phosphorus solubilising bacteria) as well as fungal load in some selected locations of Sundarban mangrove ecosystem. The relation between sediment nutrients and microbial flora were also examined during the study.

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Material and methods

Three sampling locations viz. Canning (CAN), Jharkhali (JHK) and Pakhiralay (PAK) were chosen taking in consideration of anthropogenic stress (Fig. 1). Canning and Jharkhali were situated at the bank of the river Matla where as Pakhiralay was on the river Vidhya. Canning was considered most disturbed due to anthropogenic stress.[8] The pollution load of Canning was discharged directly into Matla riverine system. Jharkhali was considered to be an intermediate between the pristine and disturbed mangrove ecosystem. At this location, restoration of mangroves was carried out at large scale by various government and non-governmental bodies. Third sampling location (near Pakhiralay) was chosen in close proximity of the core area of Sundarban mangroves. This area (Pakhiralay) was considered to be pristine and anthropogenic activities were very much restricted in this area. Water sample and sediments samples were collected in triplicates from all the locations.

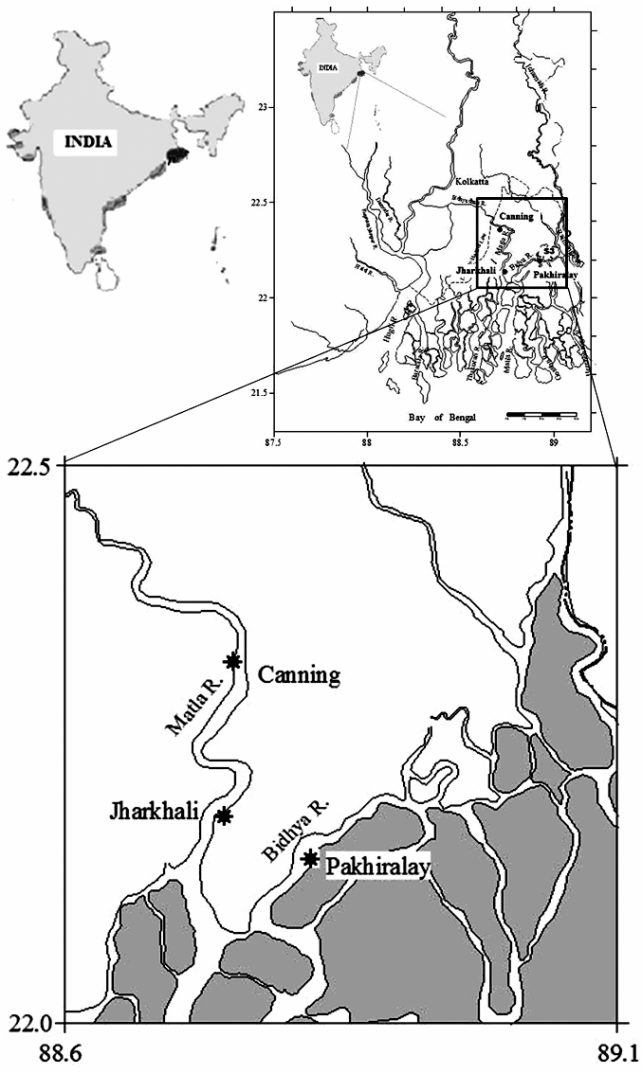

Fig. 1—Map of Sundarban mangrove ecosystem showing sampling locations

Water samples were collected in pre-washed polypropylene bottles separately for nutrient and heavy metal analysis. Samples for metal analysis were filtered through 0.45 µ glass fibre filter paper and acidified to preserve dissolve metals. The sediments were collected aseptically by carefully removing off the upper layer. The samples were kept frozen till the transfer in the laboratory where they were stored at 4°C.

Sediment samples were divided into two parts. The first part was air-dried, gently pounded and homogenized and used for granulometric and geochemical studies. Second part was immediately processed for microbial assay and nutrient analysis.

The *p*H, electrical conductivity (EC), Total dissolved solid (TDS) and redox potential (ORP) in water were measured onsite using the Thermo-Orion water analysis kit (Model Beverly, MA, 01915). Salinity was measured using refractometer. Nitrate (NO_3), bicarbonate (HCO_3), sulphate (SO_4) and chloride (Cl), in water were analyzed following the standard protocols.[11] Ammonium (NH_4) was analyzed colorimterically.[12] Sodium (Na), potassium (K) calcium (Ca) and magnesium (Mg) in the water samples were analyzed through atomic absorption spectrometer (Shimadzu, AA-680).

The granulometric separation in sediments was carried out by combining dry and wet sieving.[13] The further separation of <63 µm was done by wet sieving by Attenburg sedimentation cylinders methods based on Stokes' law. Halide free sediments were analyzed in CS analyzer (ELTRA -CS 1000) for total carbon (TC). Further the organic matter was removed[14] using 30% (v/v) H_2O_2 and the samples were used for the determination of inorganic carbon. Organic carbon (OC) was calculated by the difference Total carbon-inorganic carbon (OC = TC-IC). Total nitrogen (TN) and total phosphorus (TP) were analyzed by the Kjeldahl digestion method[15].

Five phosphorus fractions were extracted from the fresh sediments samples stepwise by a wet chemical sequential extraction (SEDEX) scheme.[16] Five sedimentary phosphorus (P) fractions were distinguished: (1) Adsorbed P (exchangeable or loosely sorbed P), (2) Oxide-P (Iron bound P), (3)

authigenic P (apatite + biogenic apatite + $CaCO_3$ bound P [CFAP]), (4) Detrital/inorganic P [FAP] and (5) Organic P. All phosphate measurements, except supernatants from step II, were done colorimeterically with the single-solution phosphomolybdate blue method.[17] Two blank (procedure blank and step blanks) were analyzed simultaneously.

Fresh sediments samples were immediately processed for the isolation of microbes. Pore-plating techniques were used for isolation of microbes from sediment samples.[18] Sterile water and labwares were used during the entire process. One gram of the fresh sediment was used to make the inoculum. The inoculum was diluted serially until 10^{-8} and 0.1 ml of the inoculum was mixed uniformly with general isolation media and mounted on the plates. The modified isolation media for bacteria[19] contained Beef Extract (3.0 gms), Becterological Peptone (5.0 gms), $NaNO_3$ (3.0 gms), KH_2PO_4 (1.0 gms), $MgSO_4$ .7 H_2O, (0.5 gms), KCl (0.5 gms), $FeSO_4$. 7 H_2O (0.01 gms), Agar (15.0 gms), Distilled water (1 L), *p*H (6.8-7.0).

Free living Nitrogen fixer has been isolated in a selective medium, comprising Mannitol (15.0 gms), K_2HPO_4 (0.5 gms), $MgSO_4$ .7 H_2O (0.2 gms), $CaSO_4$ (0.1 gms), NaCl (0.2 gms), $CaCO_3$ (5.0 gms), Agar (15.0 gms), Distilled water (1l tr) and *p*H maintained at 8.3.

Phosphate solubilising bacteria are isolated in the Pikovskayas media, containing Glucose (10 gms), $Ca_3(PO_4)_2$ (5 gms), $(NH_4)_2SO_4$ (0.5 gms), KCl (0.2 gms), Agar (20 gms), Distilled water (1 L), *p*H maintained at (6.8-7.0).

Cellulose decomposers has been isolated in selective media containing K_2HPO_4 (1.0 gms), $CaCl_2$ (0.1 gms), $MgSO_4$ .7 H_2O (0.2 gms), NaCl (0.1 gms), $FeCl_3$ (0.02 gms), $NaNO_3$ (2.0 gms), Agar (12.0 gms), Precipited Cellulose (4.0 gms), Distilled water (1 L).

Fungi isolated in the Czapedox agar media, which contained $NaNO_3$ (3.0 gms), KH_2PO_4 (1.0 gms), $MgSO_4$. 7 H_2O (0.5 gms), KCl (0.5 gms), $FeSO_4$. 7 H_2O (0.01 gms), Sucrose (30 gms), Agar (15 gms), ZnSO4.7H_2O (0.05 gms), Distilled water (1 l tr).

Isolated colonies were measured in Colony Formation Unit (CFU per gram).

$$\frac{\text{CFU}}{\text{g}} \text{ in original sample} = \frac{\text{No. of colonies counted}}{\text{(dilution factor) (volume plated, in ml)}}$$

The total fungal load was calculated in term of percentage occurrence, which may be expressed by the formula

$$\text{Percentage Occurence} = \frac{\text{No. of samples on which particular fungus is recorded} \times 100}{\text{Total number of samples examined}}$$

Statistical calculations (ANOVA and Cluster Analysis) were done using Statistica ver. 6.0.

Results and discussion

The three sampling locations differed significantly in the physiochemical characteristics of surface water. Canning was characterized by low EC (8250 mS/cm) and TDS (7250 $mg.L^{-1}$), but high ORP (135 mV). Sodium (623 $mg.L^{-1}$) chloride (957 $mg.L^{-1}$) and sulphate (612 $mg.L^{-1}$) content at Canning were also low as compared to the Jharkhali and Pakhiralay (Table 1). Phosphate (8.12 $mg.L^{-1}$) and nitrate (7.46 $mg.L^{-1}$) concentrations in the surface water were comparatively higher at Canning indicating anthropogenic inputs. In case of Jharkhali and Pakhiralay, no significant variation in various physiochemical parameters (except Silica) was observed. Hydrochemistry of these two sites was governed by tidal intrusion. The concentration of Silica at Pakhiralay was comparatively higher than that of Jharkhali. This may be attributed to higher microbial activity, which facilitated the biogenic silica mobilization (ref.). Due to prevailing reducing conditions at Pakhiralay and Jharkhali, ammonium (2.35 and 2.91 $mg.L^{-1}$) respectively was higher as compared to Canning.

Siltation was a dominant geochemical process influencing almost every riverine system of

Table 1—Physiochemical parameters of water collected from three sites at Sundarban

	*p*H	EC	TDS	ORP	SO_4	PO_4	NO_3	Cl	SiO_2	Na	K	Ca	Mg	NH_4
Canning	7.5	8250	7250	135	612	8.12	7.46	957	0.48	623	24	47	52	1.22
Jharkhali	7.4	16250	15250	118	1176	5.09	1.60	1826	2.69	1247	156	145	105	2.35
Pakhiralay	7.3	16000	15000	108	1120	4.03	2.02	1610	5.58	1236	140	140	98	1.91

All the values except *p*H EC and ORP are expressed in $mg.L^{-1}$. EC is expressed in mS/cm and ORP mV.

Sundarban.[20] Sediments collected were mostly clayey-silty in nature (Figure 2; 43-56.12% of silt content). However, in case of Pakhiralay and Jharkhali, significant quantity of clay (40.1 and 47.3% respectively) was present which may be attributed to the fact that dense network of mangrove troops act as a trap for the finer particles.[21] Ammonium was higher at Pakhiralay (678 $\mu g.g^{-1}$) and Jharkhali (612 $\mu g.g^{-1}$) as compared to Canning (488 $\mu g.g^{-1}$). Sediments were rich in organic matter (0.7-1.32%). This was to the contribution from the plant litter as well as the finer fractions of the sediments had high surface area which facilitated the adsorption of organic matter on their surfaces. No significant variation in case of total nitrogen content of the mangroves sediment was observed. High C:N ratio (Fig. 3) indicated the dominance of mineralization processes at Sundarban Mangroves.[1,21]

Total Phosphorus was observed to be more in the samples collected from the Pakhiralay (795 $\mu g.g^{-1}$) as compared to the Jharkhali (764 $\mu g.g^{-1}$) and Canning (688 $\mu g.g^{-1}$). Pakhiralay was characterized with dense mangrove forests with no human activity.[18] In a pristine ecosystem, plant litter is considered as main source of total phosphorus content of the sediments[1]. Phosphors fractions showed significant variation across the sampling locations ($F_{critical} = 3.84$; $F_{observed} = 14.41$; significance level = 0.05). Fraction 1 of phosphorus was the loosely sorbed and exchangeable phosphorus (Ex-P). It has been viewed as an intermittent form between dissolved inorganic phosphorus in the water column and inactive form in the sediment.[22] In present study, Ex-P was found to be 2-9% of the total phosphorus (Fig. 4). Significant quantity of Ex-P (9% of total P) was observed at Canning. This might be result of the direct discharge of the waste in the river Matla. Fraction 2 included oxide bound phosphorus (Ox-P) which was considered as redox sensitive, potentially mobile fraction, which is algal available.[23] Oxide bound phosphorus was highest (267 µg g-1) at Jharkhali followed by Pakhiralay (211 µg g-1). Fraction 3 represented mainly authigenic-$CaCO_3$ bound phosphorus and biogenic apatite (Au-P). Ca-bound phosphates could be usually derived from the detritus and had autogenetic origins, which may be related to the formation of calcium phosphate compounds and/or co-precipitation of phosphorus with calcium carbonate and mainly derived from digenesis. There was no significant variation observed for Au-P in case of sediment samples collected from Pakhiralay and Jharkhali. Au-P was comparatively lesser at Canning suggesting the presence of mineralization processes at the sediments associated with mangroves.[14] Fraction 4 includes the detrital apatite of igneous origin as well as other inorganic phosphorus (De-P). Identical behaviour was observed in case of Au-P and De-P. Fraction 5 represented the organic phosphorus (Org-P) of the sediments, interestingly; Org-P was the

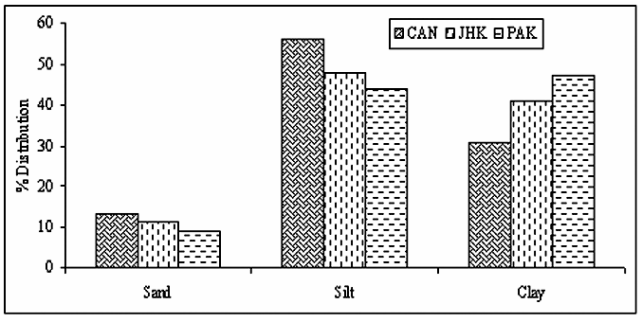

Fig. 2—Granular characteristics of three sampling locations at Sundarban

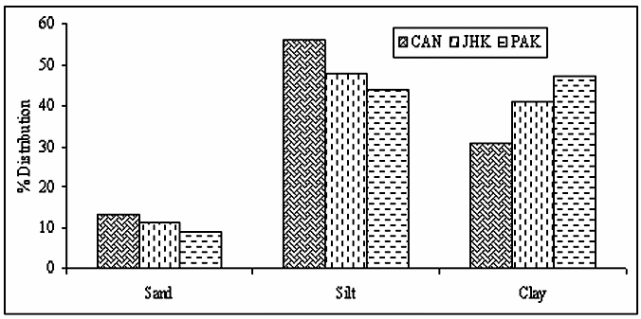

Fig. 3—C/N and Corg/Porg ratio of three sampling locations at Sundarban

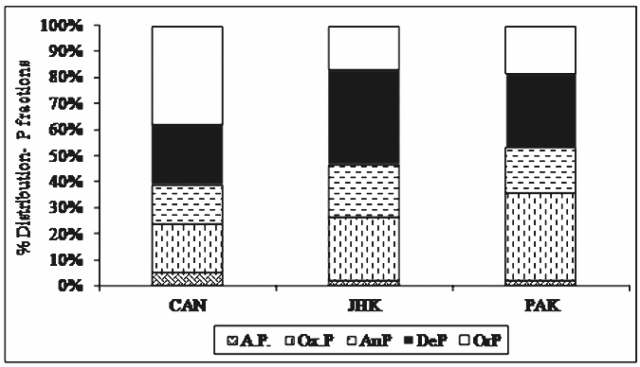

Fig. 4—Distribution of Phosphorus fraction in three sampling locations

dominant fraction for the samples collected from Canning accounting for about 40% of the total phosphorus. C_{org}:P_{org} ratio followed the order PAK>JHK>CAN (Fig. 3). Solubilisation of phosphorus is induced by microbes[24] either in the presence of *P. solubilising* bacteria or due to degradation of organic matter. At Pakhiralay, the excess of cellulose degrading bacteria as compared to other location, facilitated the decay of the plant litter, as a result the organic matter within sediment was quite high. Despite of the fact that Pakhiralay had highest Total Phosphorus, solubilisation rates were low due to lower count of *P. solubilising* bacteria.[25] The microbial diversity was assessed in the three sampling sites (Table 2) and it was observed that at Canning the bacterial load was highest (29.83×10^6 CFU.g^{-1}) as compared to Jharkhali (1.11×10^6 CFU.g^{-1}) and Pakhiralay (4.71×10^6 CFU.g^{-1}).

Table 2—Colony forming Units isolated from the samples

	Canning	Jharkhali	Pakhiralay
Nitrate forming bacteria	50.12×10^4	2.22×10^4	1.33×10^4
Free Living N_2 fixer	13.67×10^4	1.66×10^4	1.44×10^4
Cellulose degrading bacteria	4.28×10^3	8.3×10^3	45.15×10^4
Phosphate solubilizing bacteria	14.08×10^4	6.11×10^4	4.28×10^3
Total bacterial load	29.83×10^6	1.11×10^6	4.71×10^6
Total fungal load	4.1×10^4	5.15×10^3	0.64×10^4

Table 3—Table of Occurrence of fungal diversity at the three sampling location

Isolated fungal species	Canning	Jharkhali	Pakhiralay	Percentage Occurrence
Aspergillus niger	-	-	+	33.3333
A. nidulane	+	-	-	33.3333
A. orchraceous	+	+	+	100
Mucor sp.	+	-	-	33.3333
Penicillium frequentus	+	+	+	100
Penicillium sp.var 1	+	-	-	33.3333
Penicillium sp.var 2	+	-	-	33.3333
Deschirilla	-	-	+	33.3333
Botrytis	-	-	+	33.3333
Cladosporium/ Vericillium	-	+	-	33.3333
Total	6	3	5	

Nitrogen-fixing bacteria are known to improve the bioavailability of nitrogen to plants, and this capability may be enhanced when plants are also colonized by arbuscular mycorrhizal fungi.[26] Marine cyanobacteria are a particularly important component of the microbiota, constituting a source of nitrogen in every mangrove system.[27] Nitrate forming bacteria and free living N_2 fixer were also highest at Canning (50.12×10^4 CFU.g^{-1} and 13.67×10^4 CFU.g^{-1} respectively) indicating the dominance of nitrogen fixation processes. At Canning, ORP was higher as compared to Pakhiralay and Jharkhali. The existing environmental conditions facilitated the growth of nitrifying bacteria, which explained higher concentration of nitrate in overlying waters.[28]

In soil with low P bioavailability, free-living *P. solubilising* bacteria may release phosphate ions from sparingly soluble inorganic and organic P compounds in soil, and thereby contribute with an increased soil phosphate pool that may pass to pass on to the plant.[29] Available phosphorus as well as organic phosphorus at Canning was high which may be attributed to the waste discharge in the river.[18] Further *P. solubilising* bacteria (14.08×10^4 CFU.g^{-1}) were highest at Canning followed by Jharkhali (Table 2). Interestingly, Pakhiralay, the least disturbed site with highest total phosphorus concentration (795 µg.g^{-1}), had the lowest count of phosphorus solubilising bacteria (4.28×10^3 CFU.g^{-1}). In contrast, the cellulose degrading bacteria were the highest at Pakhiralay. Pakhiralay is densely covered with mangroves and excessive litter fall, optimal temperature and redox condition create an environment that is most suitable for degradation of organic matter.

Distributions of fungal species within the mangrove habitat may reflect physical conditions and/or habitat preference such as temperature, salinity, humidity, organic contents.[8] A total of ten fungal genra were identified from the sediment samples. The diversity was highest at Canning (Table 3). *Aspergillus orchraceous* and *Penicillium frequentus* were the most abundant species, reported from all the locations. *A. nigers, Deschirilla and Botrytis* was restricted to Pakhiralay only. Two other species of *Penicillium* was reported from Canning. Fungal diversity at Jharkhali was comparatively low. Apart from *Aspergillus orchraceous* and *Penicillium frequentus*, only strands of *Cladosporium* were observed.

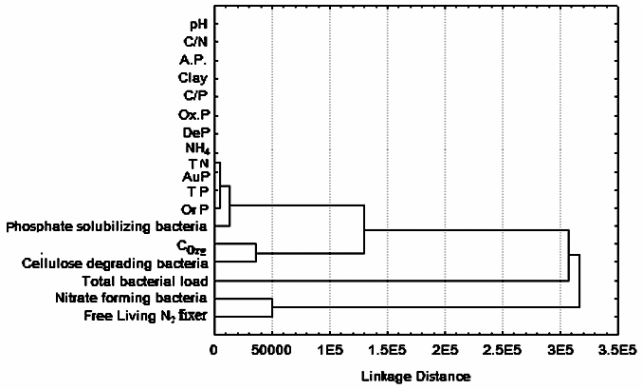

Fig. 5—Cluster Analysis of biogeochemical variables at three locations (n=9)

Analysis of Variance of the geochemical characteristics of surface water suggested significant variation across the sampling locations ($F_{critical}$ = 2.11; $F_{observed}$ = 24.47; significance level = 0.05). Similar observation were made in case of sediments samples collected ($F_{critical}$ = 1.97; $F_{observed}$ = 82.42; significance level = 0.05). However, Analysis of Variance (two ways) showed that there is no significant difference between the microbial diversity at all the sampling location. This may indicate that local environmental conditions may significantly influence the characteristics of the sampling locations. The cluster analysis of sediment geochemical characteristics (Fig. 5) was carried out for three sampling locations and it was observed that free-living N_2 fixer and Nitrate forming bacteria are clubbed in the same group indicating their inter-relationship. Cellulose decomposing bacteria's count was related to the organic carbon content of the sediments. Interestingly, *P. solubilising* bacteria showed dependency on TP, Or-P and Au-P. This may indicate that these groups of bacteria facilitate the formation of biogenic apatite and /or precipitation of phosphorus with calcium carbonate. Total microbial load didn't show dependency on any other variable.

Conclusion

The study had inferred the inter-dependency of microbes and nutrients in the sediments of Sundarban mangroves. Anthropogenic activities played an important role toward the nutrient as well as microbial behaviour. *P. solubilising* and cellulose degrading microbes were largely influenced by the nature of the sampling location. Three sampling locations were the reflectance of the microbial behaviour in mangrove ecosystem.

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