

# Spatiotemporal Analysis of Bacterial Diversity in Sediments of Sundarbans Using Parallel 16S rRNA Gene Tag Sequencing

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**Abstract** The influence of temporal and spatial variations on the microbial community composition was assessed in the unique coastal mangrove of Sundarbans using parallel 16S rRNA gene pyrosequencing. The total sediment DNA was extracted and subjected to the 16S rRNA gene pyrosequencing, which resulted in 117 Mbp of data from three experimental stations. The taxonomic analysis of the pyrosequencing data was grouped into 24 different phyla. In general, *Proteobacteria* were the most dominant phyla with predominance of *Deltaproteobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* within the sediments. Besides *Proteobacteria*, there are a number of sequences affiliated to

the following major phyla detected in all three stations in both the sampling seasons: *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Firmicutes*. Further taxonomic analysis revealed abundance of micro-aerophilic and anaerobic microbial population in the surface layers, suggesting anaerobic nature of the sediments in Sundarbans. The results of this study add valuable information about the composition of microbial communities in Sundarbans mangrove and shed light on possible transformations promoted by bacterial communities in the sediments.

**Keywords** Sundarbans · Microbial diversity · 454-amplicon sequencing · 16S rRNA gene

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## Introduction

Recent advancement in high-throughput sequencing, popularly known as next-generation sequencing technology, has immensely contributed in global increase in microbial diversity and ecology studies employing samples from different environments, e.g., soil or sediment, marine or freshwater, animal, or insect gut. Precise description of microbial diversity and abundance has recently been achieved using high-throughput sequencing platforms and explored results clearly account over 100 times more depth in the microbial diversity assessment in comparison to previous reports using the traditional culture-dependent methods [1–3]. Mangroves are among the world's most productive ecosystems, and microbes play a key role in maintaining this productivity [4]. In effect, they also constitute the largest and most diverse pool of metabolic pathways on Earth [5] with potential biotechnological and environmental implications [6]. It is estimated that about three fourth of the world's tropical and subtropical coastlines are

covered by mangrove biome. These ecosystems play numerous important roles in maintaining and sustaining the environment. They also act as buffer zones between the land and sea and are thus essential for maintenance of sea level and for protection of the coast [7]. Moreover, these ecosystems often serve as terminal basins for inland wastes and nutrient-rich sediments. The unique mangrove ecosystem serves to absorb these excess nutrients without suffering any major structural or functional disturbance [8] and, therefore, is capable of reducing eutrophication in adjacent coastal waters [8].

Mangroves are a complex and dynamic microbial ecosystem. Periodic tidal flooding, high rainfall, and humidity, together with extremely variable environmental factors such as salinity, light, temperature, and nutrient availability [5, 6] give rise to a highly diverse and adaptive microbial community in mangrove ecosystems [6, 9]. Knowledge of the microbial diversity and the activities of microorganisms in mangrove sediments are thus important for understanding how the mangrove ecosystems function. This is in turn necessary to formulate effective management and conservation strategies [6] as mangroves are already critically endangered or approaching extinction in 26 out of the 120 countries having mangroves [7].

Until recently, only a limited number of studies have attempted to analyze the microbial diversity of mangroves. Most of these studies have concentrated on the South American mangroves, which contribute to about 11 % of the total mangrove vegetation [10]. Early studies relied mostly on culture-dependent techniques, fingerprinting methods, and use of clone libraries to analyze phylogenetic and functional genes [11–16]. As a result, these studies were limited to only a small population size and did not represent the total microbial diversity. Some studies focused on the effects of pollution, oil spills, or anthropogenic stress factors on the mangrove microbial community [6, 17–21]. However, a complete snapshot of the total microbial diversity of a pristine mangrove ecosystem is presently sparse. Recently, Andreote et al. have reported a comprehensive metagenomic analysis of the Brazilian mangroves [22]. Such metagenomic studies using next-generation high-throughput sequencing techniques have the potential of providing the widest, unbiased view of the microbial diversity present in the environment [22, 23]. In addition to taxonomy and diversity, metagenomic approaches can provide a view of the potential metabolic pathways and community structures present in the environment.

The Sundarbans is the largest single block of tidal halophytic mangrove ecosystem in the world with 20,400 km<sup>2</sup> (7900 sq mi) of area covered and has been recognized as a UNESCO World Heritage site. Situated in the Ganges-Brahmaputra river delta on the Bay of Bengal, it is shared between West Bengal, India, and Bangladesh. Home to a wide variety of endangered species including the Bengal tiger, the Sundarbans play a pivotal role in protecting the coastline and averting natural calamities. However, to our knowledge, only

two studies have attempted to investigate the biodiversity of this vital and fragile ecosystem. Manna et al. attempted to correlate the different components of Sundarbans ecosystem [24], while both Ghosh et al. [12] and Chakraborty et al. [communicated] carried out small scale studies on the microbial diversity using cultivation-independent molecular approaches. Therefore, a comprehensive study of the vast microbial diversity of this unique mangrove biome is severely lacking.

In this study, we present the first detailed description of the microbes found at the surface and subsurface sediment layers in three different mangrove areas in the Indian Sundarbans. These descriptions are based on sequences of the V1–V3 region of the 16S rRNA gene as determined by 454 amplicon sequencing (pyrosequencing). Furthermore, we examined temporal changes in the microbial diversity of the sampling stations during the post-monsoon (December 2011) and monsoon (July 2012) seasons mainly to understand the role of climate and other parameters in shaping the sediment microbial communities in Sundarbans.

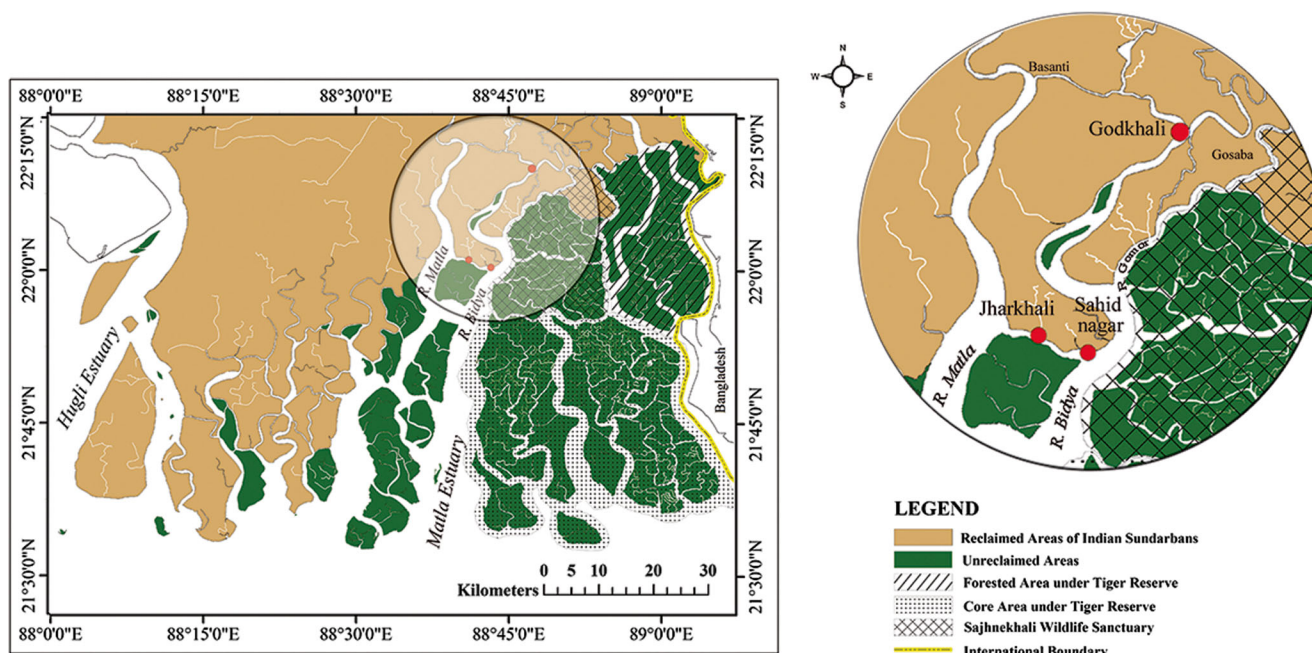
## Materials and Methods

### Study Area and Soil Sampling

Samples were taken in triplicate from surface (2 cm) and subsurface (16 cm) sediments of the Sundarbans mangrove wetland, which is located on the northeastern coast of India (Fig. 1). The samples were collected during two different seasons (post-monsoon, December 2011, and monsoon, July 2012) from three different stations, i.e., Jharkhali (station A), Sahidnagar (station B), and Godkhali (station C) along the tidal gradient in close proximity of each other (Fig. 1). The stations are representatives of different anthropogenic influences described previously. Samples were collected in triplicate from each station, and individual collection was then mixed to homogeneity to generate a representative composite sample for further analysis. Samples were brought to the laboratory in sealed sterile container, flash frozen in liquid nitrogen, and stored at –80 °C.

### Soil Analyses and Site Climate

Microbiological and biochemical analyses were performed with the field moist soils. Physical and chemical analyses were carried out with air-dried soil samples. The soil pH was measured in 1:2.5 soil/water suspensions and found to be alkaline (Table 1). The total organic carbon (TOC) was measured by the methods described previously [25, 26]. Briefly, TOC in a sample was determined by combusting the air-dried sediment sample catalytically in oxygen atmosphere into instrument chamber at 500–900 °C, and the resulting carbon



**Fig. 1** Geographical location of the sampling stations (Jharkhali—A, Sahidnagar—B, and Godkhali—C) in Indian Sundarbans. Coordinates of the sampling points and description of the stations are presented in the “Materials and Methods”

dioxide gas was detected by a non-dispersive infrared (NDIR) detector in an Aurora TOC Analyzer (O I Analytical, College station, TX, USA) that was calibrated to directly display the detected carbon dioxide mass. This mass was proportional to the TOC mass in the sediment sample and calculated as total mass of carbon per unit of sediment sample.

Conductivity and salinity were measured in situ with Hach Portable Meters (HQ40d) (Hach Company, Loveland, CO,

USA). Measured salinity was expressed in parts per thousand (ppt) or grams per kilogram, as described previously [27]. Nutrients like inorganic nitrogen (ammonia, nitrite, and nitrate), soluble phosphate, and reactive silicate were measured after quantitative extraction in respective buffering conditions following standard methodologies [28]. Briefly, nitrite was measured after complexing with sulfanilamide followed by a coupling reaction with *n*(1-naphthyl)-ethylene diaminedihydrochloride,

**Table 1** Physicochemical parameters of the post-monsoon samples (December 2011) and the monsoon samples (July 2012)

Physicochemical parameters	Station A (Jharkhali)	Station B (Sahidnagar)	Station C (Godkhali)
<b>Post-monsoon samples</b>			
pH	7.5±0.2	7.4±0.1	7.2±0.2
Salinity (PSU)	15–18	15–17	14–16
Conductivity (ms/cm)	30–36	30–34	28–32
Temperature (°C)	24±0.5	25±0.4	26±0.5
Total organic carbon (TOC) (mg/L)	AS, 8.5; A6, 8.5	BS, 5.8; B6, 12.0	CS, 2.0; C6, 7.5
Total nitrogen (N) (mg/L)	AS, 1.6; A6, 0.63	BS, 1.3; B6, 1.3	CS, 1.2; C6, 1.01
Total bacterial count (cells/g)	AS, $3 \times 10^6$ ; A6, $7 \times 10^6$	BS, $3.5 \times 10^6$ ; B6, $8.4 \times 10^6$	CS, $8.8 \times 10^6$ ; C6, $9.6 \times 10^6$
<b>Monsoon samples</b>			
pH	7.3±0.2	7.3±0.1	7.6±0.2
Salinity (PSU)	9–11	10–12	8–10
Conductivity (ms/cm)	18–22	20–24	16–20
Temperature (°C)	29±0.5	30±0.4	31±0.5
Total organic carbon (TOC) (mg/L)	AS, 7.2; A6, 8.8	BS, 4.9; B6, 9.0	CS, 1.5; C6, 6.2
Total nitrogen (N) (mg/L)	AS, 1.9; A6, 0.74	BS, 1.52; B6, 1.34	CS, 1.4; C6, 1.21
Total bacterial count (cells/g)	AS, $2.8 \times 10^6$ ; A6, $8 \times 10^6$	BS, $3.6 \times 10^6$ ; B6, $10 \times 10^6$	CS, $7.2 \times 10^6$ ; C6, $1.04 \times 10^7$

PSU practical salinity unit

which forms an azo dye upon coupling. The resulting azo dye was measured spectrophotometrically at 543 nm. The nitrate, in contrary, was quantitatively reduced to nitrite using cadmium (Cd) granules prior to measurement. The total nitrite was then measured spectrophotometrically as described earlier, and further subtraction of the measured value of free nitrite in the sediment resulted in determination of nitrate in the sample. Ammonia was measured in a reaction with hypochlorite under alkaline condition, which results in formation of monochloramine. In a successive reaction with phenol and nitroprusside, monochloramine was converted into indophenol blue, which was measured spectrophotometrically at 630 nm. The soluble phosphate was measured using acidified molybdate reagent, which yields phosphomolybdate complex upon reaction with soluble phosphate. This complex has further reduced into molybdenum blue and measured spectrophotometrically at 880 nm. The reactive silicate was measured using the formation of yellow silicomolybdic acid in the presence of molybdate under acidic condition (Figure S1).

#### Soil DNA Isolation

Soil DNA was extracted from 0.5 g soil subsamples using the MoBio DNA Power Soil kit (MoBio Laboratories, Carlsbad, CA). A NanoDrop (Thermo Scientific, Wilmington, DE, USA) spectrophotometer was used to obtain an accurate quantification of the extracted DNA and to measure other important parameters for DNA quality, such as the ratio of absorbance at 260:280 nm and 260:230 nm. Isolated soil DNA with a ratio of absorbance at 260:280 nm and 260:230 of 1.8 and 2.0, respectively, was used to initiate amplicon sequencing analysis.

#### PCR Amplification of Bacterial 16S rRNA Gene

Amplification of the V1–V3 region of the 16S rRNA gene was performed in a 25- $\mu$ l reaction mixture containing 17.50  $\mu$ l of water (Invitrogen, USA), 2.5  $\mu$ l Fast Start buffer (10 $\times$ ), 0.5  $\mu$ l dNTP (10 mM), 1.0  $\mu$ l Fusion Forward Primer (10  $\mu$ M), 1.0  $\mu$ l Fusion Reverse Primer (10  $\mu$ M), 0.5  $\mu$ l fast start enzyme (5 U/ $\mu$ l fast start high PCR system, Roche), and 2  $\mu$ l extracted soil DNA. The fusion primers consisted of a template-specific sequence and a fixed sequence, as recommended by the “Sequencing Technical Bulletin No. 013-2009” (454 Life Sciences, USA). In the 5'–3' direction, forward primers contained a Roche A sequencing adaptor (CCATCTCATCCCTGCGTGTCTCCGAC), a key sequence (TCAG), a 10-bp Multiplex Identifier (MID) sequence, and a template specific sequence GAGTTTGATCCTGGCTCAG. Reverse primers contained a Roche B sequencing adaptor (CCTATCCCCTGTGTGCCTTGCCAGTC), a key sequence (TCAG), and a template-specific sequence ATTACCGCGGCTGCTGG. The PCR reactions were run on a Veriti Thermal

Cycler (Applied Biosystem) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 45 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. Samples were held at 4 °C.

#### Library Preparation

PCR amplicons were evaluated by electrophoresis on a 1.5 % agarose gel. The amplicon library was purified with Agencourt AMPure XP beads (Beckman Coulter Inc., Canada) and quantified by fluorometry using the Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen, Burlington, ON) according to the Roche 454 “Amplicon Library Preparation Method Manual” of the GS Junior Titanium Series (454 Life Sciences, USA). Pooled amplicons were diluted as recommended and amplified by emulsion PCR on a Thermal Cycler 9700 (Applied Biosystems) according to the Roche 454 “em-PCR Amplification Method Manual—Lib L” (454 Life Sciences, USA). Pyrosequencing was performed for 200 cycles on a Roche 454 GS-Junior sequencing instrument according to the manufacturer's protocol (454 Life Sciences, USA). All reads were filtered using the standard read rejecting filters of the GS Junior sequencer, namely key pass filters, dot and mixed filters, signal intensity filters, and primer filters (454 Sequencing System software Manual, V 2.5.3, 454 Life Sciences, USA).

#### 454 Data Processing and Analysis

Analysis of the pyrosequence data was carried out using a combination of mothur (software version 1.33.0) and Ribosomal Database Project (RDP-II). The raw data were subjected to initial quality trimming using the mothur software [29], and all reads having an average quality value of <20 were discarded. Then, we removed the number of chimeric sequence by using UCHIME algorithm. Then, the chimeric free reads were further screened for the presence of ambiguous bases, and any reads which have a length <200 were discarded and forward primer sequence was removed from the final dataset. The obtained processed reads were then demultiplexed to separate sample based on the 10-bp MID sequence, and any reads which do not match to the MID were discarded. The high-quality reads were then aligned to bacterial 16S rRNA gene sequence, and clustering was performed at 97 % similarity using the RDP pipeline; a representative sequence from each cluster was selected based on abundance using the sequence selection tool. The representative sequences obtained from each cluster were then classified using the naive Bayesian Classifier (Ribosomal Data Project, release 10) at a bootstrap confidence of 80.

To assess the dissimilarities between the bacterial communities in the given surface and subsurface sediment samples in two seasons, non-metric multidimensional scaling (NMDS)



was performed. NMDS plots for communities using Jaccard and Sorenson similarity indices (jclass and sorclass) were created using the NMDS algorithm in *mothur* [29].

In addition, sample dispersion based on various environmental parameters was evaluated through principal component analysis (PCA) using Past v 2.17c software [30]. Prior to the multivariate analysis, standardization of the entire dataset was carried out by subtracting the mean from each observation and divided by the corresponding standard deviation. The standardized dataset was also used to generate a cluster of the samples according to the environmental variables using Euclidean distance as the measure of similarity between samples.

#### Nucleotide Sequence Accession Numbers

All 454-GS Junior sequence data from this study were submitted to the NCBI Sequence Read Archive (SRA) under accession numbers: SRR1032105 ( $A_{\text{Surface\_Dec11}}$ ), SRR1221694 ( $B_{\text{Surface\_Dec11}}$ ), SRR1231523 ( $C_{\text{Surface\_Dec11}}$ ), SRR1060346 ( $A_{\text{Subsurface\_Dec11}}$ ), SRR1224602 ( $B_{\text{Subsurface\_Dec11}}$ ), SRR1231530 ( $C_{\text{Subsurface\_Dec11}}$ ), SRR1523729 ( $A_{\text{Surface\_July12}}$ ), SRR1224601 ( $B_{\text{Surface\_July12}}$ ), SRR1231528 ( $C_{\text{Surface\_July12}}$ ), SRR1060363 ( $A_{\text{Subsurface\_July12}}$ ), SRR1224603 ( $B_{\text{Subsurface\_July12}}$ ), and SRR1231524 ( $C_{\text{Subsurface\_July12}}$ ).

## Result and Discussion

### Description of Sampling Sites

The Sundarbans mangrove along the Bay of Bengal has evolved as deltaic formations through natural deposition of upstream sediments by three major rivers: Ganges, Brahmaputra, and Meghna and their tributaries. Other major rivers flowing through this ecosystem are Saptamukhi, Thakuran, Vidyadhari, Matla, and Goasaba. The geosystem in Sundarbans is dominated by deltaic formations that are made up of a mosaic of low-lying “islands” intersected by a complex network of tidal waterways, estuaries, creeks, and mudflats. The Sundarbans floor varies from 0.9 to 2.11 m above sea level. The average temperature ranges between 20 and 40 °C, with average high during May–June and low in December–January, respectively. Rainfall is heavy with humidity as high as 80 % as it is close to the Bay of Bengal. The subtropical monsoon lasts from June to September with an average rainfall of 125.5–221.07 mm/month during the monsoon period. Samples were collected in December 2011 (with an average rainfall of 5.4 mm/month) and July 2012 (with an average rainfall of 293.6 mm/month). The pH was found comparable in both the sampling seasons. Previous studies have shown that the salinity in Sundarbans gradually

increased from post-monsoon (17.3 PSU) to pre-monsoon periods (24.5 PSU) and reached its lowest value during the monsoon (12.6 PSU) season. Within the water column, dissolved oxygen (DO) concentration was found to be high during post-monsoon period and low in monsoon season [24].

In the present study, two sampling stations are located in Jharkhali Island (Fig. 1). This island is surrounded on the three sides by the Matla and the Vidyadhari rivers and by a creek, Boro Herobhanga Khal, joining these two river flow. This creek remains flooded by water coming from both rivers particularly during high tide and monsoon season. The area is dominated by typical mangrove habitat. Station A (Jharkhali jetty; 22° 01' 07.6139" N, 88° 40' 56.1910" E) is located in the subtidal area at the southern part of the Jharkhali island and on the bank of Boro Herobhanga creek. This station is inundated by sea water throughout the year. This station consists of low-density mangrove vegetation mostly dominated by Hental Palm (*Phoenix paludosa*). Due to the presence of the jetty in this sampling station, the area is under anthropogenic pressure mainly due to oil leakage and agri-waste disposal from the inhabited area within the island. Station B (Sahid Nagar; 22° 00' 25.5999" N, 88° 42' 13.9438" E) is located near the subtidal preserved area on the southeastern side of the Jharkhali Island and at the junction of Vidyadhari river and Boro Herobhanga creek. This area is in the process of eco-restoration because of the large-scale successful afforestation program. This area is densely vegetated with mangrove plant species like Hental Palm (*P. paludosa*), Sundari (*Heritiera fomes*), Gewa (*Excoecaria agallocha*), Goran (*Ceriops decandra*), and Keora (*Sonneratia apetala*). Little anthropogenic influences are present in station B, mainly due to human activities and sedimentation events and could be considered as nearly pristine mangrove sediment in the present study. Station C (Godkhali; 22° 06' 32.57" N, 88° 46' 22.22" E) is located on opposite side of the Gosaba Island (Fig. 1), which is the last inhabited island towards the Sajnekhali Wildlife sanctuary and Sundarbans Tiger project area of Sundarbans reserve forest (UNESCO World Heritage site: 1989). This station is situated in an intertidal zone with high anthropogenic influences mainly due to the addition of industrial effluents and oil leakage. The Commercial and touristic use of the Jetty situated near this station further influence the flora and fauna in the neighborhood. Mangrove plants are scarce in this area, and non-mangrove plant species dominate the floral counterpart indicating that the typical mangrove ecosystem has been altered in this area.

### General Statistics

To our knowledge, this is the first report of the microbial diversity analysis in the Sundarbans sediment using pyrosequencing. We analyzed bacterial 16S rRNA gene (SSU) amplicons prepared from 12 sediment samples originated

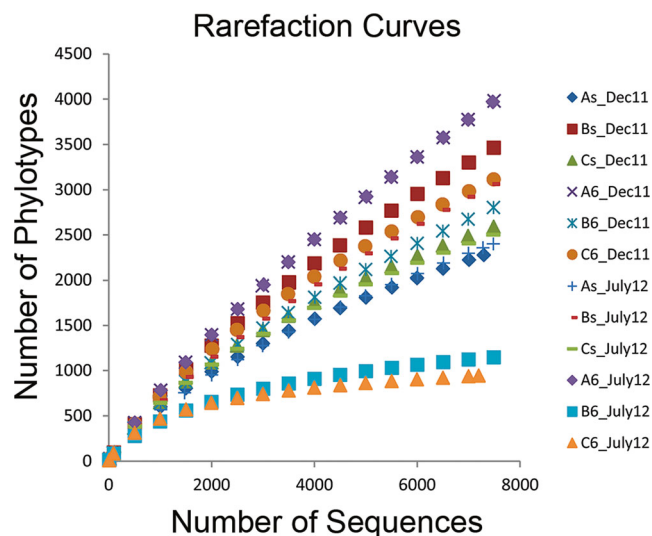
from three sampling stations at two different depths and sampled in two seasons, i.e., post-monsoon (December 2011) and monsoon (July 2012) respectively. We generated a pyrosequencing dataset containing 2,24,413 sequences with an average length ranges between 494 and 530 bases, which add up to a total of 116.98 Mb (Table S1).

#### Estimation of Richness and Diversity

The maximum operational taxonomic units (OTUs) detected across the sampling stations according to the observed clusters (sorbs) at 3 % dissimilarity was 8766 (Table 2) in the subsurface sample from station A, Jharkhali, during the post-monsoon season (December 2011). The maximum amount of OTUs observed is also reflected in the Chao values predicted for the sample (Table 2). Though the higher values of OTUs corroborated well with the estimated Chao index, the richness values were not necessarily followed similar trends. In general, for the surface samples collected during the sampling seasons (December 2011 and July 2012), Chao and Shannon indices showed marginal differences. However, for subsurface samples, the species richness decreased from December 2011 to July 2012. The rarefaction curves indicated similar profiles of all samples (Fig. 2). We also normalized the data considering the same number of sequences to all samples (Table 2), and we observed clear seasonal differences between the subsurface samples collected from three stations. To this end, our diversity analysis revealed that despite of several changes in the physico-chemical parameters (salinity, conductivity, and water level) during the monsoon season compared to previous post-monsoon season, little changes appeared in the microbial diversity and richness at the surface levels in all three sampling stations. However, a continuous decrease in both microbial diversity and richness at the subsurface samples during the seasonal shift from post-monsoon to monsoon

**Table 2** Estimated OTU richness and diversity indices for 16S rRNA libraries of sediment of mangrove samples

Sample ID	Number	Cluster	Chao index	Shannon index
A <sub>Surface</sub> _Dec11	11,544	2959	5711.98	6.90
B <sub>Surface</sub> _Dec11	10,303	4037	11,945.09	7.56
C <sub>Surface</sub> _Dec11	12,186	3411	6444.97	7.13
A <sub>Subsurface</sub> _Dec11	22,607	8766	22059.0	8.11
B <sub>Subsurface</sub> _Dec11	18,491	5103	12019.89	7.30
C <sub>Subsurface</sub> _Dec11	31,208	7388	15510.24	7.74
A <sub>Surface</sub> _July12	21,543	4622	9236.25	6.83
B <sub>Surface</sub> _July12	20,793	6081	13727.18	7.46
C <sub>Surface</sub> _July12	16,183	3870	7070.82	7.21
A <sub>Subsurface</sub> _July12	8248	901	1047.63	5.86
B <sub>Subsurface</sub> _July12	8712	1202	1538.12	6.10
C <sub>Subsurface</sub> _July12	7295	945	1149.29	6.25



**Fig. 2** Rarefaction curves of partial sequences of 16S rDNA

possibly exemplifies the attainment of continuous anaerobic environment at the subsurface sediment levels during the monsoon season.

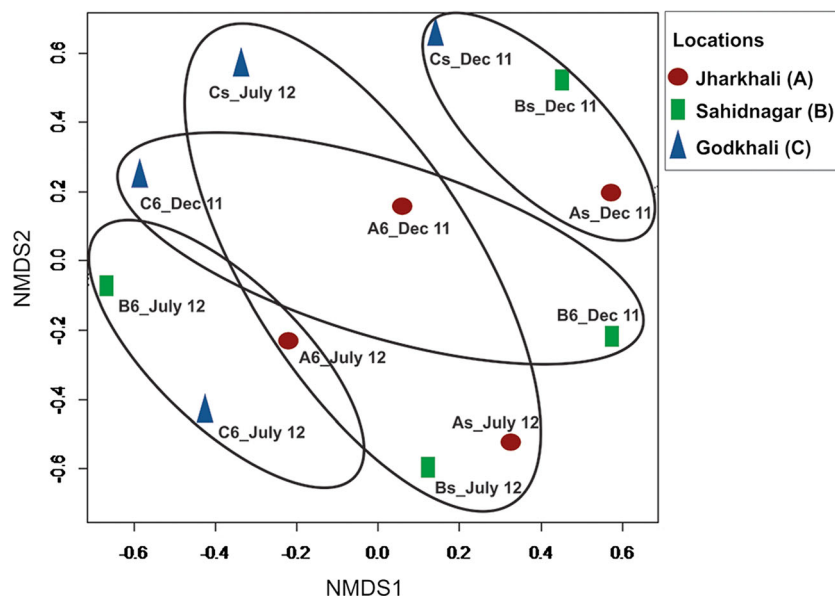
Further, we have performed NMDS to compare the community composition using presence/absence data. NMDS employs an iterative algorithm that optimizes the position of samples into a low-dimensional space minimizing the difference between rank-order of multivariate ecological dissimilarity and distances in NMDS space (Fig. 3). From the figure, it is easily observed that despite the low temporal variation in the relative abundance of different phyla, surface and subsurface communities from different sampling stations were more closely related to each other during a season (monsoon/post-monsoon), with only exception of Cs\_July11 sample [Godkhali (C)], than to samples from surface and subsurface levels of a station in either of the seasons or between surface and subsurface samples from two seasons.

In order to examine the temporal variations of the environmental parameters in the sampling stations, we performed a principal component analysis (PCA) on all environmental data using PAST. Environmental parameters assessed for each composite sample representing either the surface or subsurface origin has been summarized in Table 1. The PCA biplot analysis reveals a distinctive pattern of the environmental parameters recorded in the present analysis (Figure S2). Salinity, conductivity, total organic carbon (TOC), and temperature are distributed along the PC1 axis (54.06 %) whereas pH and total nitrogen (TN) are predominantly dispersed along the PC2 axis (16.29 %) (Figure S2).

#### Bacterial Diversity in Mangroves Based on SSU rRNA Genes (Taxonomic Description and Inventory)

A total of 2,24,413 representative sequences for SSU rRNA were present in the dataset, with the values of 38,968, 77,836,

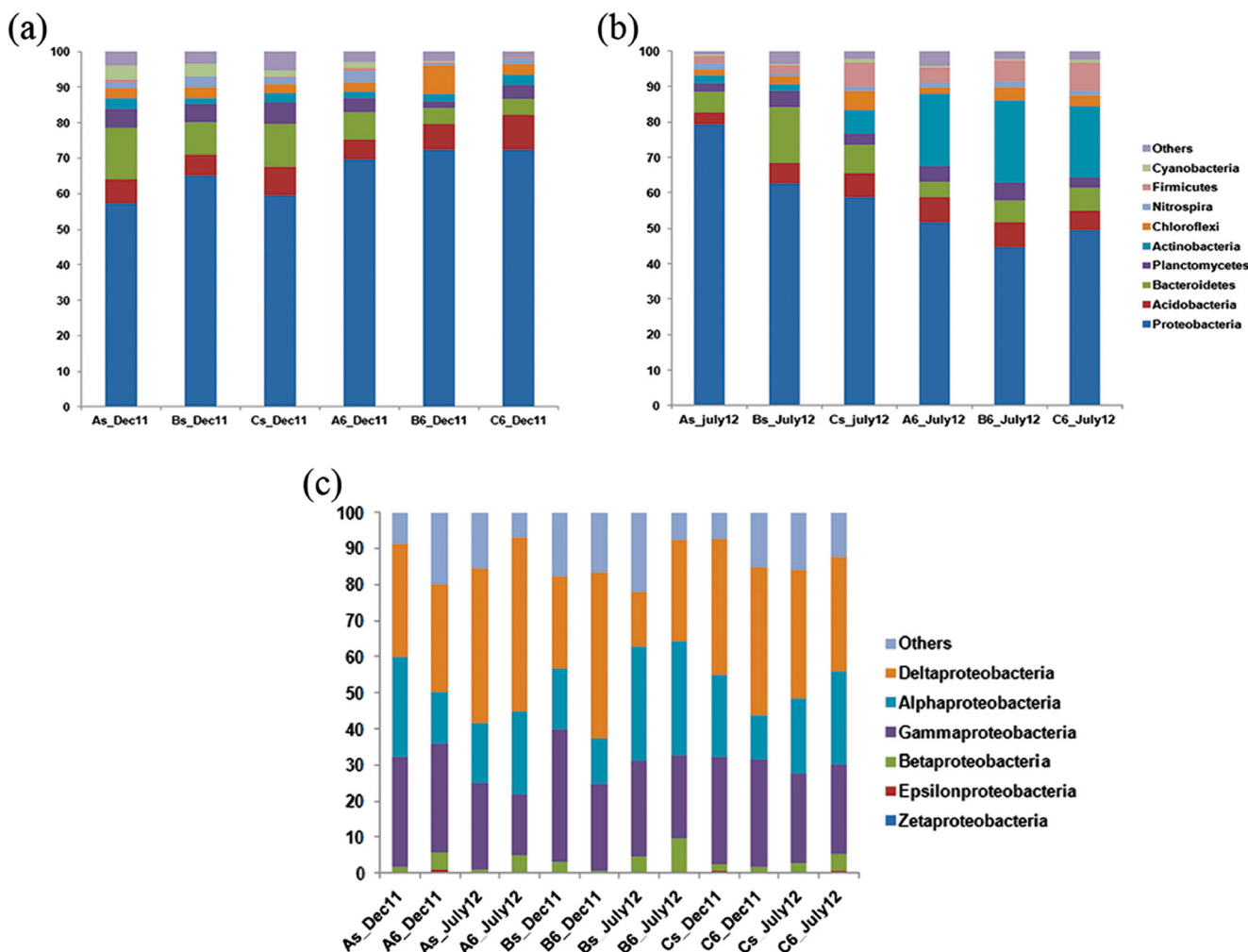
**Fig. 3** Non-metric multidimensional scaling plot of all samples studied (surface and subsurface samples of three sampling stations, e.g., A, B, and C in December 2011 and July 2012, respectively). Pairwise abundance-based Jaccard indices were used to construct the plot



67,397, and 40,212 for Surface\_Dec 2011, Subsurface\_Dec 2011, Surface\_July 2012, and Subsurface\_July 2012, respectively (Table S1). The analysis of the sediment samples from all three stations in two seasons using pyrosequencing grouped the sequences into 24 different phyla. In general, *Proteobacteria* were the most dominant phyla that account for about 57–72.4 % in December 2011 and 49–79 % in July 2012 samples, respectively, of the total number of phyla detected in all three stations (Fig. 4, Table S2). In the mangroves, higher abundance of *Proteobacteria* has been reported using different molecular approaches previously [18, 22]. Furthermore, capillary sequencing-based molecular studies in Sundarbans mangrove revealed the dominance of *Proteobacteria* within the sediment bacterial community [12]. Besides *Proteobacteria*, there are a number of sequences affiliated to the following major phyla detected in all three stations in both the sampling seasons: *Actinobacteria* [1.57–3.12 % in December 2011 and 1.9–23.2 % in July 2012], *Bacteroidetes* [4.23–14.26 % in December 2011 and 4.27–15.62 % in July 2012], *Planctomycetes* [2.06–6.08 % in December 2011 and 2.43–5.46 % in July 2012], *Acidobacteria* [5.45–9.91 % in December 2011 and 3.41–7.07 % in July 2012], *Chloroflexi* [2.53–8.10 % in December 2011 and 1.48–5.42 % in July 2012], *Cyanobacteria* [0.09–4.01 % in December 2011 and 0.10–0.97 % in July 2012], *Nitrospira* [0.32–3.11 % in December 2011 and 0.71–1.75 % in July 2012], and *Firmicutes* [0.06–1.06 % in December 2011 and 2.30–7.96 % in July 2012] (Figure 4a, b; Table S2). Among the distinct sediment samples collected in two seasons (post-monsoon, December 2011, and monsoon, July 2012), the following differences were observed: Higher abundance of *Proteobacteria* was evident in the surface communities in monsoon season and subsurface samples in post-

monsoon season in all the three stations; higher abundance of *Bacteroidetes* in the surface communities was evident in all three stations in both the season; *Firmicutes* were more abundant in these stations in monsoon samples with comparatively higher in numbers in the subsurface samples; *Actinobacteria* were higher in numbers in the subsurface samples collected from these stations in monsoon and finally a comparable abundance of *Cyanobacteria*, *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, and *Nitrospira* was evident in three stations in both the seasons (Fig. 4a, b). Candidate phyla *Gemmatimonadetes*, *Chlorobi*, *Spirochaetes*, *Verrucomicrobia*, *Fusobacteria*, *Deferribacteres*, *Armatimonadetes*, *Tenericutes*, *Deinococcus-Thermus*, *Lentisphaerae*, TM7, OD1, OP11, BRC1, and WS3 were represented by comparatively less number of sequences detected in the sediment samples. A full list of bacterial taxonomic data can be found in the supplementary table S2. The taxonomic groups found and their relative level of occurrence found comparable to the data obtained by Andreote et al. [22] and Dos Santos et al. [18], who assessed the diversity of bacteria in the tropical mangrove sediments of Brazil.

Phylogenetic analysis of the identified proteobacterial sequences revealed a common distribution pattern of the classes in all three stations in the present study. The most frequent classes detected were *Deltaproteobacteria* (except for station B), followed by *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Epsilonproteobacteria* (Fig. 4c). In both pristine mangrove sediments and mangroves under anthropogenic influences, dominance of the classes *Deltaproteobacteria* and *Gammaproteobacteria* was recorded previously using 16S rRNA gene tag sequencing technique and metagenomics [18, 22]. In the tropical mangrove sediments located on the coast of Sao Paulo state of Brazil, Andreote et al. [22] have performed metagenomic analysis



**Fig. 4** Composition of different phyla based on the classification of partial rRNA gene sequences of bacteria from Sundarbans sediments using RDPII classifier (v 2.2). **a** Distribution of phyla in post-monsoon

(December 2011) sediments, **b** Distribution of phyla in monsoon (July 2012) sediments. **c** Differential proportion of sequences assigned within the phylum *Proteobacteria*

where they detected higher abundance of the classes *Deltaproteobacteria* and *Gammaproteobacteria*. Furthermore, Dos Santos et al. [18] have used pyrosequencing of 16S rDNA tags to show dominance of these group of proteobacterial classes in the sediments of “Restinga da Marambaia” mangrove in Rio de Janeiro, Brazil, under natural conditions and after simulated oil spills. Taken together, bacterial community analysis of the tropical mangrove sediments revealed a universal dominance of the proteobacterial classes *Deltaproteobacteria* and *Gammaproteobacteria* both under pristine conditions and under anthropogenic interference. The higher abundance of *Deltaproteobacteria* in sea water and in marine sediments has been proposed to be similar to the mangrove ecosystems, where frequent anaerobic conditions facilitate in selection of microaerophilic/anaerobic sulfate-reducing organisms belonging to deltaproteobacterial class [20]. In general, oxygen penetration in the sediment remains limited to the upper few centimeters, and the subsurface sediment beneath the surface is shown to be anaerobic in

nature. Predominance of *Deltaproteobacteria* at the surface and subsurface sediment samples collected in the present study clearly demonstrates the anaerobic nature of the sediments both at the subtidal and intertidal regions of Sundarbans especially during the monsoon season.

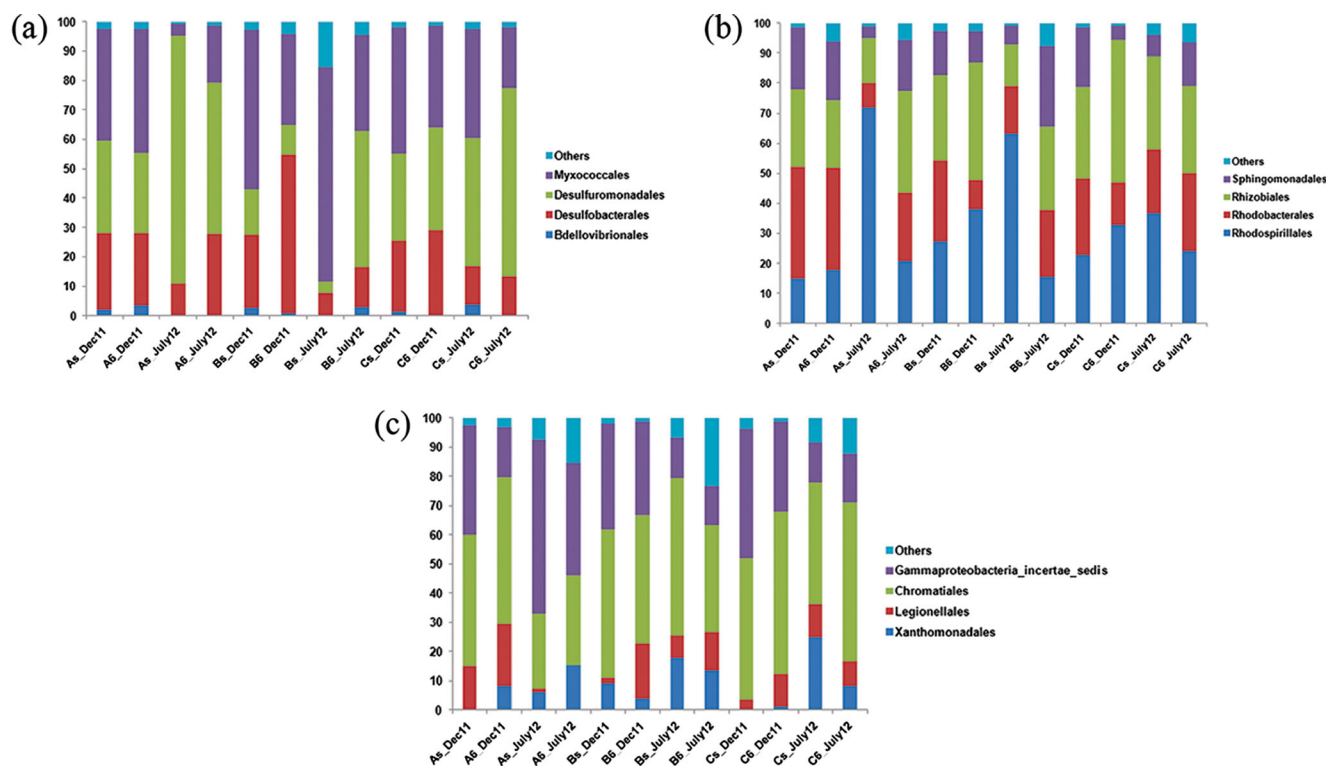
The *Deltaproteobacteria* were dominated by orders *Desulfobacterales*, *Desulfuromonadales*, *Myxococcales*, and *Bdellovibrionales*. Abundance of *Desulfobacterales*, *Desulfuromonadales*, and *Bdellovibrionales* in the sediment samples collected from three stations in general indicates anaerobic nature of the sediment. Previous studies have shown that the *Deltaproteobacteria* were dominant due to anaerobic condition of the mangrove sediment that drives selection of specific microbial groups such as sulfur/sulfate-reducing bacteria belonging to phylum *Deltaproteobacteria* [20]. In the present study, the *Myxococcales*, representing the only aerobic deltaproteobacterial order, found mostly abundant in the surface-derived samples from station B in both the seasons indicates comparatively less anaerobic nature of the samples



collected from this station. To this end, we believe that the station B (Sahidnagar), which is under eco-restoration, has sufficient mangrove vegetation that further influenced the microbial abundance and diversity, as evident from the present dataset. It has been proposed that the activities of the mangrove roots provide a source of oxygen and interfere directly to the redox potential of the mangrove sediment [31]. Due to eco-restoration, station B might receive more available oxygen compared to other two stations. In contrary, in both stations A (Jharkhali) and C (Godkhali), vegetation has been heavily affected due to anthropogenic influences, such as oil leakage and addition of agri-industrial wastes, which ultimately reduced the number of mangrove trees and most importantly not all the mangrove plant species are present in these stations. This scenario might have led to higher abundance of anaerobic sulfur-reducing bacteria such as *Desulfobacterales*, *Desulfuromonadales*, and *Bdellovibrionales* in stations A and C, respectively.

After *Deltaproteobacteria*, *Gammaproteobacteria* were the most abundant proteobacterial class in all the three stations in post-monsoon samples (Fig. 4c). However, in monsoon samples, *Gammaproteobacteria* and *Alphaproteobacteria* both shared equal abundance after *Deltaproteobacteria* in these stations (Fig. 5a). Neither depth dependent (spatial) nor any seasonal differentiation was observed with respect to the abundance of *Gammaproteobacteria* in the sampling stations. Within our dataset, the *Gammaproteobacteria* were

dominated by the orders *Chromatiales*, *Legionellales*, and *Xanthomonadales* (Fig. 5c). *Chromatiales* includes a group of anaerobic bacteria, commonly known as purple sulfur bacteria, which are capable of photosynthesis by oxidizing hydrogen sulfide to sulfide and sulfate. *Chromatiales* are commonly reported in anoxic lake sediments and in other aquatic habitats including mangrove sediments and anoxic conditions are primary pre-requisites for their sulfur-photolithoautotrophy [32]. In contrary, members of the order *Legionellales* are not commonly encountered in mangrove sediments and they comprised two families, *Legionella* and *Coxiella*, both are involved in human disease [33]. Abundance of *Legionellales* in Sundarbans sediments clearly indicates human intervention in this ecosystem. Besides these orders of *Gammaproteobacteria*, a number of sequences affiliated to the orders *Pseudomonadales*, *Enterobacteriales*, *Methylococcales*, *Alteromonadales*, *Oceanospirillales*, and *Thiotrichales* were identified within the present dataset. In their study on Brazilian mangrove sediments, Dos Santos et al. [18] have shown that several orders within the *Gammaproteobacteria* show major shift after oil contamination. Especially, the order *Chromatiales* was shown to be sensitive as it was dominant before oil contamination and decreased significantly after oil contamination. Within Sundarbans sediments, however, we could see a constant dominance of the order *Chromatiales* in all three sampling stations with different history of anthropogenic activities



**Fig. 5** Composition of different classes based on the classification of partial 16S rRNA sequences of *Proteobacteria* from Sundarbans sediment using RDPII classifier (v 2.2). Deltaproteobacterial orders (a), *Alphaproteobacteria* orders (b), and *Gammaproteobacteria* orders (c)

possibly confirming the fact that although oil and hydrocarbon contaminations are evident in Sundarbans, especially in stations A and C, alteration of the abundance of an order requires considerable concentration of those contaminants to be added into the Sundarbans mangrove sediment systems. Besides, no representatives of the orders *Alteromonadales* and *Oceanospirillales* were detected in the present study. These gammaproteobacterial orders were shown by Dos Santos et al. [18] to be dominant after oil contamination in the Brazilian mangrove sediment.

*Alphaproteobacteria* were dominated by the orders *Rhodospirillales*, *Rhodobacterales*, *Rhizobiales*, and *Sphingomonadales* (Fig. 5b). Among the alphaproteobacterial orders, *Rhodospirillales* was the most abundant and recorded to be maximum in the monsoon dataset from stations A (71.7 %) and B (63.1 %). In general, the order *Rhodospirillales* is represented by two families, e.g., photosynthetic non-sulfur *Rhodospirillaceae* and heterotrophic acetic acid synthesizer, *Acetobacteraceae*. Besides *Rhodospirillales*, *Rhizobiales* representing nitrogen fixing and symbiotic bacteria, *Rhodobacterales* representing primary colonizer on the temperate ocean sediments and coastal water columns [34], and *Sphingomonadales* representing the aromatic hydrocarbon-degrading bacteria [35] were the dominant alphaproteobacterial orders detected in all three stations in both the seasons. Besides the dominant alphaproteobacterial orders, a number of sequences affiliated to the orders *Caulobacterales*, *Kordiimonadales*, *Rickettsiales*, *Sneathiellales*, and *Kiloniellales* were detected within the present dataset.

Betaproteobacterial sequences were dominated by the orders *Burkholderiales* and *Rhodocyclales*. In Sundarbans sediment, members of the order *Burkholderiales* with diverse functional attributes have been reported previously [12, 36]. Most of the identified members of *Rhodocyclales* order include organisms that reside in aquatic habitat and prefer oligotrophic conditions. Besides these orders, *Nitrosomonadales*, *Hydrogenophilales*, *Methylophilales*, and *Neisseriales* were also detected with the dataset.

Actinobacterial sequences were detected in all the three stations in both the seasons under study. Interestingly, actinobacterial sequences are more abundant in the subsurface samples in monsoon samples. This might be due to the adsorption of *Actinobacteria* to the mineral particle surface, and the salinity in such micro-niches was lower than what was measured for the subsurface sediment samples. However, the diversity was found lower at the surface confirming the ecological principle that more extreme environment, e.g., salinity in this case, affects the diversity of *Actinobacteria*. Furthermore, during the monsoon season, an increased addition of the freshwater/rain water through the major rivers in this area further reduces the local salinity in these sampling stations (approx. 12 PSU). The phylum *Actinobacteria* was dominated

by orders *Actinomycetales* and *Acidimicrobiales*. Besides, members of the following orders were also detected in limited number with the sediment: *Solirubrobacterales*, *Rubrobacterales*, *Nitriliruptorales*, *Coriobacterales*, *Euzebyales*, and *Spaerobacterales*. *Actinomycetales* was found highly abundant at the subsurface samples in the monsoon season. Previous analyses on diversity of *Actinobacteria* in saline sediments revealed detection of *Acidimicrobiales* in low-salinity sediments and absence in higher salinity sediment samples [37]. In Sundarbans sediment, the observed salinity remained in the lower side (12–20 PSU) possibly due to the addition of continuous freshwater and this has further suggested the reason for domination of halo-tolerant *Acidimicrobiales* in this sediment. Previous culture-dependent analyses in Indian Sundarbans mangrove have identified and characterized several salt-tolerant antibacterial *Streptomyces* spp. [38].

*Acidobacteria* were found evenly distributed in all three sampling stations in both seasons. Previous studies have shown that phylogenetic evenness for *Acidobacteria* largely depends on the soil pH [39, 40]. The relative abundance was shown strongly correlated with the soil pH, with *Acidobacteria* representing larger portion of soil bacterial communities at low pH soils. In Sundarbans sediments, pH remains nearly neutral and uniform throughout the year (pH 7.3–7.8). This has further corroborated with the findings of even distribution of *Acidobacteria* in these sampling stations. In marine ecosystems, the abundance of *Acidobacteria* has been reported previously [22, 41]. In the Red Sea, for example, *Acidobacteria* were reported in the middle and deep layers of coral reef sands where suboxic and anoxic conditions are being maintained [41]. Such findings not only supported acidobacterial distribution in Sundarbans but also correlated well to the findings that the sediments of Sundarbans are suboxic in nature.

Representative sequences affiliated to candidate phyla *Planctomycetes*, *Bacteroidetes*, *Nitrospira*, *Chloroflexi*, *Cyanobacteria*, and *Firmicutes* were ubiquitous in all three stations both at the surface and at subsurface levels of the sediments. *Planctomycetes* sequences were represented by classes *Phycisphaerae* and *Planctomycetacia*. *Bacteroidetes* sequences were dominated by the members of the classes *Flavobacteria* and *Sphingobacteria*, which corroborate well with our previous culture-independent capillary sequencing-based analysis of the bacterial population in these three stations [communicated]. Among the *Firmicutes*, *Bacilli*, and *Clostridia* orders were dominating in all the stations. *Chloroflexi* and *Nitrospira* sequences were dominated by orders *Anaerolineae* and *Nitrospira*, respectively. Only a few cyanobacterial sequences were detected in each of these sampling stations, and most importantly, we could not assign cyanobacterial sequences below the phylum level.

The number of sequence reads related to candidate divisions TM7, WS3, OD1, OP11, and BRC1 was detected in the sediment samples from both seasons. To date, no cultivated representatives exist representing either TM7, WS3, or OD1 candidate divisions. Thus far, no distinct ecological attribute has been assigned to members of the candidate division TM7 that are commonly found in a wide range of environments, including terrestrial (rhizosphere, soils, and peat bog) and aquatic (groundwater, freshwater, sea water, and deep-sea sediments) habitats [42]. Members of the candidate division WS3 were detected only in the methanogenic environments [43] and OD1 division is widespread in environments dominated by sulfur, such as marine environments and sulfureta [42]. OP11 division is commonly detected in terrestrial and marine environments, and recent partial genome sequencing analysis of one of the members belonging to this division revealed several eco-physiological aspects in this candidate division [44, 45]. Candidate division BRC1 was originally detected in extreme, low-diversity environments, and the ecological role of this candidate division is still yet to be unraveled [42].

## Conclusion

This is the first 16S rRNA gene amplicon sequencing analysis of microbial diversity of Sundarbans, the unique tiger land mangrove of the world in the backdrop of climate change. The massive parallel 454 amplicon sequencing of 12 samples of the surface and subsurface sediments and data analysis unraveled the complexity of the bacterial diversity in the sediment samples. Furthermore, detected diversity of the bacterial community composition has clearly provided novel insights into the ongoing biogeochemical processes associated with the regular deposition of sediment in this coastal mangrove. Moreover, this study also unfolds the bacterial community structure during seasonal changes between permanently inundated sediments and sediments submitted to seasonal dewatering. To this end, an increased understanding of microbial community variation with respect to seasonal changes rather raise the questions of whether this observed composition changes during monsoon and post-monsoon would confer a better resistance to the subsequent drying stress for microbial communities in seasonally dewatered sediments or such changes reflected an alteration of microbial structure with respect to changes in the biogeochemical cycling of nutrients. Further analyses, however, are essential for better understanding of the sediment biogeochemical cycles and their relationship to the microbial community pattern in the sediment. This work is also important in building a database and an understanding of how microbial communities vary geographically and with anthropogenic intervention.

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