

Microbial community structure of surface sediments from a tropical estuarine environment using next generation sequencing

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ABSTRACT

Microbial community structure was analyzed from tropical monsoon influenced Mandovi-Zuari (Ma-Zu) estuarine sediment by means of Next Gen Sequencing (NGS) approach using Ion Torrent PGM™. The sequencing generated 80,282 raw sequence reads. Barcoding with Ion Tags allowed multiplex analysis of microbial community and helped in identifying shifts in microbial community structure. Analysis of sequence data revealed that sediment at both the stations in the Mandovi estuary was dominated by Archaeal group, *Euryarchaeota* (53.1% and 64.01%). Among *Euryarchaeota*, *Methanomicrobia* was dominant. *Methanococci* was present only at the mouth and *Methanopyri* was detected at the mid-estuarine station. Whereas, both the stations of Zuari estuary were dominated by Bacteria, *Proteobacteria*, mainly *Gammaproteobacteria* (97.67% and 54.41%). A clear influence of mangrove ecosystem on the bacterial diversity was evident in the Zuari estuary. These results suggest that the two estuaries have a very distinct microbial community structure. Characterization of microbial communities in this study area using NGS for the first time points out that even within geographically close habitats, the microbial population structure is significantly influenced by localized interactions. The signatures obtained from sediments can thus be used to reconstruct habitat characteristics and serve as biomarkers. Future studies should focus on the functional gene profiling of different microbial communities and the influence of seasons and tide in such monsoon influenced estuaries.

Keywords: Metagenomics, Next Gen Sequencing (NGS), Microbial diversity, Sediment, Mandovi-Zuari (Ma-Zu) estuarine complex, Ion Torrent PGM™.

1. Introduction

Estuarine systems have dynamic and diverse bacterial communities owing to the mixing of fresh water and sea water and are altered by autochthonous biological activity (Crump et al., 2003). The Mandovi and Zuari estuaries are tropical, tide dominated, monsoon influenced estuaries located in the south-eastern Arabian Sea. Although both these estuaries are adjacent to each other, they are influenced by different factors. Mandovi estuary receives greater run-off than Zuari owing to many tributaries feeding terrestrial inputs into this estuary (Qasim, 2003), and is also narrower and experiences more frequent navigational activities when compared to Zuari. Mandovi estuary receives an influx of nutrients, especially nitrates (Sardessai and Sundar, 2007), the sources of which can be attributed to terrestrial inputs, the mangrove swamps (Qasim and Gupta, 1981) and from the discharge of iron ore extraction-plants at the upstream end (De Souza et al., 2003). Due to an increased mining activity in both the estuaries, high concentrations of iron and manganese have been reported (Kessarkar et al., 2015). They also receive heavy sewage outfalls adding both organic and inorganic inputs.

Although few studies report the distribution of various pathogenic, indicator as well as limno-tolerant and halo-tolerant bacteria in the water column (Divya et al., 2009; Nagvenkar and Ramaiah, 2009; Rodrigues et al., 2011; Khandeparker et al., 2015), the work on the microbial community in the sediments is sparse. Sediments act as a repository of the events occurring in the pelagic environment and the processes occurring and the communities present in the surface sediments have a profound effect on the local and global cycling of elements (Arrigo, 2005). Recently, Singh et al. (2010) reported the Archaeal community distribution through phylogenetic analyses using 16s rRNA gene with an emphasis on the *AamoA* gene in the sediments of these estuaries. Despite their ubiquity, relatively less is known about micro-organisms, largely because more than 99% of prokaryotes in the environment cannot be cultured in the laboratory and show resistance to culture under standard laboratory conditions, a phenomenon that limits the understanding of microbial physiology, genetics and community ecology (Woese et al., 1990). Among the different methods used to characterize and unravel the genetics of uncultured microorganisms, the genomic analysis of a population of microorganisms (metagenomics), has emerged as a powerful tool which involves direct isolation of genomic DNA from an environment (Schloss and Handelsman, 2005; Tringe and Rubin, 2005). Both the Polymerase Chain Reaction (PCR) based analysis of 16S rRNA and shotgun metagenomic studies have been used to characterize soils (Fierer et al., 2011), oceans (Caporaso et al., 2011), and the atmosphere (Bowers et al., 2011).

Ion Torrent PGM™, a newly introduced next-generation benchtop sequencing platform uses sensor array chips that can monitor millions, and potentially billions, of simultaneous sequencing reactions (Merriman et al., 2012). Recently, Whiteley et al. (2012) assessed the bacterial and archaeal dynamics within covered anaerobic digesters used to treat piggery wastes using Ion Torrent PGM™. It is clear from their study that the PGM platform provides a low cost, scalable and high throughput solution for both Tag sequencing and metagenomic analyses (Whiteley et al., 2012). Yergeau et al. (2012) also applied the Ion Torrent technology to 16S rRNA-based profiling of complex bacterial communities of the Athabasca river.

In the present study, the microbial community in the sediments of two different tropical estuaries was evaluated using the Ion Torrent Personal Genome Machine (PGM) as microbial community characterization using culturable methods and phylogenetic analyses using 16s rRNA gene provide partial information, whereas metagenomics is comprehensive and holistic. For this study, two stations from Mandovi estuary viz., Campal and Panjim and two stations from Zuari estuary viz., Chicalim and Siridao were selected. It was hypothesized that the diversity in the physico-chemical conditions and the dissimilarities in the degree of anthropogenic influence at these two different estuarine ecosystems would reflect on the microbial communities prevailing in their respective environments. To the best of our knowledge this is the first study in this region.

2. Materials and Methods

2.1. Site description and sampling

Mandovi and Zuari estuaries have been described as the lifelines of Goa, originate in the Western Ghats and flow through a narrow coastal plain and along the west coast of India and are extensively used in transportation, fisheries and recreational activities. They differ in their geomorphology, rainfall pattern and complex estuarine ecosystem. Both estuaries are of nearly identical length (~50 km each), are highly productive and dynamic systems and have wide mouth regions and longer flushing periods. In this study, Chicalim (15°24'10.92"N, 73°51'8.55"E) and Siridao (15°25'41.89"N, 73°52'38.84"E) were sampled in the Zuari estuary (Fig. 1). Chicalim (Zu-Ch), is highly influenced by anthropogenic activity and shipping industry when compared to Siridao which receives inputs from the mangrove area and is relatively pristine (Zu-Si). High numbers of pathogenic bacteria have been previously reported in this area (Nagvenkar and Ramaiah, 2009; Rodrigues et al., 2011; Khandeparker et al., 2015). Campal (15°29'36.26"N, 73°48'42.08"E) and Panjim (15°30'9.76"N, 73°50'10.24"E) in Mandovi estuary were selected as sampling stations (Fig. 1). Panjim is a mid-estuarine station (Ma-Pa), receives inputs from different anthropogenic activities as it is close to the

city centre and large number of fishing trawlers, whereas, Campal is situated at the mouth of the estuary (Ma-Ca), receives lesser inputs other than the influx carried towards this location through currents and tides. All the sediment samples were collected using van Veen grab during pre-monsoon season (Zuari – April 2013; Mandovi – March 2014) in sterile 50 ml falcon tubes and transported to the laboratory on ice for DNA extraction and stored at -20°C until further processing.

2.2. Sample processing and DNA isolation

Sediment DNA extraction was performed using Ultraclean Soil DNA Kit, (MoBio lab. Geneworks, Australia). The DNA was extracted using bead beating and column purification which was performed according to the manufacturer's guidelines. The metagenomic DNA was quantified by Eppendorf-Biospectrometer, and run on 0.8% agarose gel. The gel was viewed using Bio-Rad Gel Doc™ EZ Gel documentation system after staining with Ethidium Bromide (EtBr). Metagenomic DNA was stored at -80°C until further downstream processing.

2.3. PCR based analysis using Ion tags

All chemicals for PCR viz., DNA polymerase, deoxynucleotide triphosphates (dNTPs), Taq DNA polymerase, nuclease free water, 10X reaction buffer, MgCl₂ were purchased as PCR Core Kit with Taq DNA Polymerase from Sigma Aldrich, USA.

V6 hyper variable region of bacterial 16S rRNA gene was amplified by PCR as described in Sogin et al. (2006) using A-967F (5'- CAACGCGAAGAACCTTACC-3') and B-1046R (5'- CGACAGCCATGCANACCT-3') primers. PCR amplification mix contained 5 units of Taq DNA polymerase, 1X reaction buffer, 200µM dNTPs and 0.2 µM concentration of each primer in a volume of 100 µl. Genomic DNA (3–10 ng) was added to two separate 50-µl amplification mixes. Cycling conditions included an initial denaturation step at 94°C for 3 min; 30 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min; and a final 3 min extension step at 72°C. PCR amplification of the PCR products were processed in duplicates, were checked for size and specificity by electrophoresis on 2% w/v agarose gel.

DNA concentration was assessed on Qubit high sensitivity assay kit (Invitrogen, Life Technologies). PCR amplicons, which were amplified in duplicates, were pooled in an equimolar concentration. Prior to sequencing, library preparation, end repair, adaptor ligation was done according to the protocol specified by the manufacturer. PCR purification (Agencourt AMPure XP beads, Beckman Coulter) and quantification (Qubit 2.0 Fluorometer) was performed in between each step. Subsequently, the samples were adjusted to a final concentration of 25-30pM and pooled in an

equimolar concentration. The pooled amplicons were attached to the surface of Ion Sphere Particles (ISPs) using an Ion Xpress Template 100bp (base pairs) kit (Life Technologies, USA). Enrichment of total ISPs resulted in >80% templated-ISPs which was sequenced on the 314' (10 Mega bases) micro-chips on the Ion Torrent Personal Genome Machine (Life Technologies, USA) for 65 cycles (260 flows) using Ion Express Template 100bp chemistry resulting in an expected average read length of >100 bp. The Ion Torrent PGM has an inbuilt software plugin capability to filter low quality reads, polyclonal sequences and automatic trimming of the sequences matching the PGM 3' adapter (adapter trimming).

Filtered PGM data was exported as Standard Flowgram Format (SFF) files and uploaded on the Ribosomal Database Pipeline (RDP), a pyrosequencing pipeline (<https://pyro.cme.msu.edu>). Before trimming, both reverse and forward primers in the sequences were retained. The RDP initially sorts low quality sequences, trimming off the key tags, primers and minimum length sequences ≤ 50 bp (Cole et al., 2013). Trimmed "Fastq" files containing quality reads were converted to fasta (sequence), qual (quality score), flow (flowgram) files using Mothur version v.1.36.1 (Schloss et al., 2009) by command line "fastq.info (fastq=sample.fastq)". All quality sequences "fasta" files were checked for chimeric sequences by UCHIME reference algorithm (Edgar et al., 2011) where reference sequence data was used from Silva Database and removed for subsequent analysis (0.4% removed). Trimmed "Fastq" files containing quality reads were subjected to similarity based hierarchy in The Ribosomal Database Project (RDP) Naive Bayesian rRNA Classifier (Version 2.10) (Wang et al., 2007) where the confidence threshold was 50% to allow maximum read coverage.

Data obtained in the form of reads were visualized as pie charts using Golden Software Grapher™ 8. To summarize the considerable amount of information obtained about the species distribution in the metagenomes of different ecosystems, cluster analysis was done using the similarity profile routine (SIMPROF) test in Primer software (Clarke and Warwick, 2001). The reads were initially standardized, log transformed and clustered based on resemblance using Bray – Curtis similarity.

3. Results

3.1. DNA extraction, PCR amplification, read output and base positional quality from 314 chip

The extracted metagenomic DNA was pure and showed no fragmentation or lysis of DNA (Fig. 2a). After PCR amplification, all samples showed no contamination or non – specific amplification and yielded pure, sufficient quantity of amplicons (Fig. 2b).

Subsequent to sequencing, ISPs with libraries were 47.7% & 46.0% respectively, after exclusion of the polyclonal, low quality, primer dimer library ISPs in the Zuari and Mandovi samples (Table 1). The absolute number of PCR amplified 16sRNA gene read outputs were quantified and subjected to quality based filtration using a pre-installed plugin in the Ion Torrent Server.

The data predicted on the Ion 314™ Chip is 10 mega bases. The total sequences obtained on the chip for metagenomes were 80,282. All sequences were further trimmed on RDP where ≤ 50 bp sequences were discarded. After trimming, 47,030 quality sequences were used for classification.

3.2. Sediment metagenome at Mandovi estuary

The mouth (Ma-Ca) as well as the mid-Estuarine (Ma-Pa) stations were dominated by domain Archaea. The abundance was high at the Ma-Ca in which it contributed to 99.06% of the metagenome as compared to 77.4% in the Ma-Pa station. However, Bacteria comprised of only 0.94% to 1.47% of the metagenome at both the stations (Fig. 3c and d).

Phylum *Euryarchaeota*, under domain Archaea, which includes methanogens, some halobacteria and thermophilic aerobes and anaerobes, contributed to 53.1% and 64.01% of the metagenome at Ma-Ca and Ma-Pa respectively. But 0.3% of the metagenome belonged to phylum *Crenarchaeota* which includes the major extremophiles of the marine environment, at the Ma-Ca compared to 3.7% at Ma-Pa. The sediment metagenome at the Ma-Pa was comparatively more diverse owing to the presence of *Thaumarchaeota* (1.32%), unclassified *Thaumarchaeota* (0.45%) and other classes (0.04%) (Fig. 4c and d).

In the class wise distribution, class *Methanomicrobia* was dominant, but with varying contribution to the metagenome (67% at Ma-Ca and 85.75% at Ma-Pa). *Methanococci* contributed to 7.21% at the estuarine mouth, Ma-Ca while they were not detected at the mid-estuarine station Ma-Pa. However, *Methanopyri* (1.58%) was present in the sediments of Ma-Pa station which was not detected at Ma-Ca. *Thermoprotei* and unclassified *Thermoprotei* together contributed 7.0% in Ma-Ca while class *Thermoprotei* alone contributed 9.84% at Ma-Pa (Fig. 5c and d). In general, it is clear that the diversity was distinctly different at the mouth and the mid-estuarine station.

3.3. Sediment metagenome at the Zuari estuary

A significant difference was observed in the sediment metagenome of Zuari estuary when compared to Mandovi estuary. The sediment community was dominated by Bacteria (96.67% and 40.59% at Chicalim (Zu-Ch) and Siridao (Zu-Si) respectively when compared to Archaea in the Mandovi estuary. Domain Archaea was not observed at Zu-Ch whereas at Zu-Si, it constituted less than

0.01%. Unclassified reads of Bacteria were observed in both Zu-Ch and Zu-Si stations, but in varying percentages (3.33% and 29.4% at Zu-Ch and Zu-Si respectively) (Fig. 3e and f).

Phylum wise distribution revealed a significant variation in the metagenomes in both the stations in Zuari estuary, although preserving and elucidating the difference in the community structure in Ma-Zu estuarine complex. Majority of the metagenome was comprised of *Proteobacteria* (97.67%) at Zu-Ch and other classes were only 2.33%. Whereas, at Zu-Si station, although class *Proteobacteria* were dominant (54.41%), the rest of the community comprised of class *Actinobacteria* (26.41%), unclassified *Acidobacteria* (7.76%), *Cyanobacteria* (5.724%) and *Bacteroidetes*, unclassified *Proteobacteria* (<2%) (Fig. 4e and f).

The soil metagenome at Zu-Ch was dominated by *Gammaproteobacteria* (91.0%), which comprises of all the major pathogenic groups in the marine microbiome, whereas unclassified *Gammaproteobacteria* and other classes comprised of 7.17% and 1.83% respectively. *Gammaproteobacteria* were the dominant class at Zu-Si but only 55.93% as compared to 91.0% at Zu-Ch. The remaining metagenome also showed significant variation with the remaining metagenome at Zu-Si comprising of unclassified *Acidobacteria* (Grp. 22) (17.53%), unclassified *Gammaproteobacteria* (12.97%), Chloroplast (6.68%) and *Acidobacteria* (Grp. 10) and *Sphingobacteria* (<4%) (Fig. 5e and f).

3.4. Cluster Analysis

Cluster analysis using SIMPROF test revealed two distinct clusters at 60% similarity cut-off in domain wise clustering, 40% cut-off in phylum wise clustering and 50% cut-off in class wise clustering. One cluster belonged to the metagenome at Zuari estuary and the other to Mandovi estuary (Fig. 6).

4. Discussion

4.1. Significance of microbes in estuaries

Mandovi and Zuari are tropical monsoon influenced estuaries although adjacent to each other provide diverse physico-chemical conditions. The authors examined the metagenomes of the sediments in the Ma-Zu estuarine complex to gain a holistic view of the bacterial community structure in the sediments using next generation sequencing. To our knowledge this is the first study to characterize and compare the sediments simultaneously at both the estuaries. Microbes play a vital role in the microbial loop, thus influencing food web dynamics and ecosystem functioning, and are influenced by the physical processes. The organic matter (dissolved or particulate) that is transported

from the rivers to the estuaries is the key component of biogeochemical cycles of many estuarine systems (Hopkinson et al., 1998; Raymond and Bauer, 2000). The decomposition of organic matter by microbes is a key process of carbon cycling in aquatic ecosystems (Stern et al., 2007). The world's oceans harbour different microbial communities of Bacteria, Archaea, protists, and unicellular fungi which account for most of the oceanic biomass. These microscopic factories are responsible for 98% of primary production (Atlas and Bartha, 1986; Whitman et al., 1998) and mediate most of the biogeochemical cycles in the oceans. With each new investigation, this window on the microbial world increases in size and previously unknown microorganisms are discovered, many of which have major impacts on oceanic processes (Sogin et al., 2006).

In the present study, a cost-effective, rapid and scalable NGS technology was used to unravel the uncultivable novel microbial communities. The capability of high throughput sequencing of 16S rRNA gene sequences by means of Next Generation Sequencing (NGS) technologies has been pivotal in facilitating the discovery of micro biota biodiversity (Whiteley et al., 2012). Prior to the advent of NGS, the high throughput genetic analysis of complex microbial community samples was only possible using low resolution 'fingerprinting' technologies (Griffiths et al., 2011), Sanger sequencing at extremely high cost (Rusch et al., 2007) or pyrosequencing (Sogin et al., 2009), DGGE techniques which have comparatively lower outputs and reliability. In the present study area, recently Fernandes et al. (2014) used pyrosequencing technique to gain insights on the taxonomic diversity of benthic bacterial community in the tropical mangrove systems in the Mandovi estuary.

4.2. Sediment phylogenetic composition at Mandovi estuary

The sediment of both the stations in the Mandovi estuary was dominated by Archaeal groups (*Euryarchaeota*), mainly by *Methanomicrobia*. Initially, it was thought that Archaea inhabit only extreme environments, however, recent studies have indicated their presence in different environments, including fresh water lakes and continental shelf sediments (Vetriani et al., 1999; Glissman et al., 2004). Archaeal activity in the sediment has lots of potential to describe the active biogeochemical cycles as nitrification, sulphur metabolism, methane oxidation and methanogenesis. Archaea in particular play an important role in decomposition of organic matter (ammonification) and denitrification. In the present study, Phylum *Euryarchaeota*, contributed to 53.1% and 64.01% of the metagenome at Ma-Ca and Ma-Pa respectively. But 0.3% of the metagenome belonged to phylum *Crenarchaeota*, at the Ma-Ca compared to 3.7% at Ma-Pa. *Euryarchaeota* and *Crenarchaeota* phyla are widespread in the oceans, play an important role in carbon and nitrogen cycles (Ingalls et al., 2006; Francis et al., 2007) and are especially abundant in the estuaries (Zeng et

al., 2007). Non-extremophilic *Crenarchaeota* have been reported to possess the ammonia mono oxygenase (*amoA*) gene (Francis et al., 2005). Ammonia Oxidising Bacteria (AOB) and Ammonia Oxidising Archaea (AOA) are among the dominant groups in the ocean sediments and play an important role in utilizing the fixed ammonia from the system (Wuchter et al., 2006).

The shallow estuarine sediments in Pearl River estuary, Southern China were dominated by *Crenarchaeotal* groups (Jiang et al., 2011). The core samples from this region revealed the presence of methanotrophs (mainly *Euryarchaeota*) in all sediment layers. *Methanomicrobia* belong to methanogens and have renowned importance in the carbon cycle through methanogenesis in anoxic marine sediment and sulfate reduction (Bhattacharyya et al., 2015). Methanotrophs play an important role in anaerobic oxidization of methane and thus control the emission of methane (Alperin et al., 1988; Orphan et al., 2001; Ye et al., 2009). They are also vital for mineralization of organic matter in anoxic sediments (Saia et al., 2010). The ubiquity of methanotrophs is evident from various studies which have reported their contribution to the benthic community structure of deep sea anaerobic sediments (Vetriani et al., 1999), hot springs or deep sea hydrothermal vents (De Long, 1992), Crater lake sediments (Antony et al., 2012) and shallow estuarine sediments (Jiang et al., 2011). The biogeochemical roles of Archaea especially the methane metabolizing microorganisms in deep sea environments has been well studied. Phylogenetic analysis have revealed remarkably high diversity of Archaeal assemblages in the estuaries from temperate and tropical environments (Abreu et al., 2001; Vieira et al., 2007; Zeng et al., 2007; Singh et al., 2010). *Euryarchaeota* and *Thaumarchaeota* have been reported to be more abundant planktonic Archaeal groups in the water column (Lincoln et al., 2014) but their ubiquity in the sediments is not reported in this region. Xie et al. (2014), observed an increase in *Thaumarchaeota*, containing hyperthermophilic organisms, with increasing salinity. However, in the present study, we have observed their presence in Panjim station in the Mandovi estuary, which is upstream of Campal station, mouth of the estuary suggesting that salinity alone does not play a major role in the distribution of Archaeal groups and may be influenced by the presence of nutrients among other factors. Consistent with the previous reports, sediments of Mandovi estuary are dominated by methanotropic Archaea (Singh et al., 2010).

Saia et al. (2014) reported the presence of methanogens in tropical estuarine sediments which are highly contaminated, especially with pollutants of petroleum source. To the best of our knowledge the ubiquity and functional role of methanogens in tropical estuarine sediments and their response to various anthropogenic inputs in such environments is barely reported in this region. Active anaerobic metabolism results in proliferation of methanogenic Archaeal community in sediment (Vieira et al., 2007). The abundance of methanogens (*Methanomicrobia*, *Methanobacteria* and

Methanopyri) was higher in Ma-Pa station, which is comparatively more influenced by anthropogenic activities than Ma-Ca. High amount of anthropogenic activity is reported in the Mandovi estuary through mining and navigational activities, resulting in accumulation of petroleum hydrocarbons (PHCs) in the sediment (Veerasingam et al., 2015) and in different body parts of sea snake such as gut, liver and kidney (Mote et al., 2015).

4.3. Sediment phylogenetic composition at Zuari estuary

The sediment of Zuari estuary was dominated by Bacteria mainly *Proteobacteria*, and was enriched with *Gammaproteobacteria*. Since the sediment of Mandovi estuary was dominated by Archaea, it seems that these two estuaries have a very distinct community structure and thus ecosystem functioning. *Gammaproteobacteria* includes most of the pathogenic groups such as *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* which are known to grow under wide range of salinity conditions and are mostly introduced by anthropogenic inputs. Earlier studies on cultivable bacteria in Zuari estuary showed dominance of *Vibrio*, *Alteromonas*, *Enterobacter*, *Marinobacter*, *Aeromonas* and *Exiguobacterium* (Khandeparker et al., 2011; Fernandes et al., 2014; Khandeparker et al., 2015). The use of lipid molecules to identify the organic matter sources in these estuaries suggested the contribution of organic matter to be from both natural and anthropogenic sources. Organic matter in the Mandovi estuary is mainly from terrestrial sources followed by petroleum sources. A comparatively higher contribution of organic matter from petroleum origin was reported in the sediments near Zuari estuary (Harji et al., 2008). This difference in the allochthonous organic matter inputs had a profound effect on the microbial community composition.

Siridao is an estuarine beach located 2km upstream from the mouth and on the northern bank of the Zuari estuary (Ingole and Parulekar, 1998). The salinity at this place is usually lower than surrounding areas and the sediment texture has been reported to be sandy-silty. Dissolved Oxygen concentrations are lower than the other surrounding areas (Alkawri and Ramaiah, 2010). This area is influenced by mangrove ecosystem as compared to Chicalim. This explains a more diverse community structure at Siridao which showed the presence of diverse phylotypes (Zhang et al., 2009). Our study is consistent with the previous work carried out by Fernandes et al. (2014), showing the presence of *Acidobacterial* groups among other phylotypes in sediments influenced by mangrove ecosystem. Culture-independent approach in mangrove sediments in Sao Paulo, Brazil showed dominance of *Proteobacterial* and *Acidobacterial* groups (Dias et al., 2010). *Acidobacteria* are ubiquitous in soil bacterial communities (Jones et al., 2009). Moreover, *Acidobacteria* Gp10,

which is reported in the present study, is previously stated to be abundant in marine sediments (Orcutt et al., 2011). *Proteobacterial* communities were detected to represent a majority of the clone library in the surface sediments of sub-tropical mangrove habitats (Liang et al., 2007). Similar results have been reported before in coastal marine sediments (Bowman and McCuaig, 2003; Asami et al., 2005). Our results indicated an increase in the *Gammaproteobacteria* as compared to previous studies which reported a dominant *Deltaproteobacterial* community in the mangrove swamps of Zuari estuarine system (Bharathi et al., 1991). This might be due to the nutrient availability and change in the oxygen availability as anoxic environments are dominated by *Deltaproteobacteria* (Schwarz et al., 2007).

Although majority of the metagenome of Zuari estuary comprised of class *Proteobacteria* (54.41%), at Zu-Si station the rest of the community was mainly comprised of class *Actinobacteria* (26.41%), unclassified *Acidobacteria* (7.76%), *Cyanobacteria* (5.724%) were also present. The estuarine habitat at the Chesapeake Bay revealed large numbers of *Alphaproteobacteria* in regions where salinity was low. *Beta* and *Gammaproteobacteria* were also present in both the areas. *Cyanobacteria* and *Epsilonproteobacteria* were detected in a few locations (Affourtit et al., 2001). *Actinobacteria* is the next most abundant phylum which is of significant economic importance (Ward and Bora, 2006) and is known to contain groups which can produce antibiotics (Adinarayana et al., 2006; Kim et al., 2006; Manivasagan et al., 2009) and also play a part in cellulose degradation (Pankratov et al., 2006; Khandeparker et al., 2011). Marine *Actinobacteria* are known to produce unique and wide range of antibiotics (Charan et al., 2004; Sujatha et al., 2005). It is reported that around 45% of the bio active compounds are from *Actinomycetes* (Berdy, 2005). Antibiotics are produced by marine bacteria as a response to nutrient depleted conditions, limited space for growth or competition from rival strains (Slattery et al., 2001). This supports the findings of our work, where *Actinobacteria* were prevalent in Zu-Si station where diversity was higher.

Recently, Wang et al. (2012) evaluated microbial community in marine sediment, freshwater and intertidal sediment communities using millions of Illumina reads along Pearl River, China. They demonstrated that freshwater sediment was dominated by *Acidobacteria*, and *Proteobacteria* (*Alpha* and *Beta*), whereas intertidal sediment had *Chloroflexi*, *Bacillariophyta*, *Gammaproteobacteria*, *Epsilonproteobacteria*, *Actinomycetales*, *Bacteroidetes* and *Firmicutes*.

Chicalim (Zu-Ch) area is surrounded by various shipping industries and the Zuari estuary that drains into the Chicalim bay receiving significant anthropogenic inputs. Thus, there is a substantial risk of pollution impact on the ecosystem in this area. Additionally the Zuari estuary and the Cumbarjua

canal which feed water into the bay, are extensively used for transport of manganese and iron ores (Kessarkar et al., 2015), which affect the fragile ecosystem that resides at Chicalim bay. Sediment texture at Zu-Ch station has been reported to be comprised of high silt clay content and fine but poorly sorted grain size (Ansari et al., 1993). Bacterial diversity has been reported to be low in sediments prone to heavy metals (Qing et al., 2007) or hydrocarbon contamination (Greer, 2010). The stress conditions resulting from high concentration of heavy metals, favours the proliferation of particular bacterial communities, which explains the less diverse community at Chicalim. Also, the increased anthropogenic impact in such environments has been reported to have detrimental effects on human health (Nogales et al., 2011). *Gammaproteobacteria* dominated the sediment community at Chicalim. *Gammaproteobacteria* play an important role in sulphur cycling and have been reported to be important members of the prokaryotic community in coastal marine sediments (Asami et al., 2005). They also form the most abundant denitrifiers in the marine sediments (Bhatt et al., 2005). The bacterial community composition also varies with varying abundance of benthic forms to which they are associated with (Gihring et al., 2009). Some microbial taxa have been reported to catalyse phytodetritus degradation and denitrification in coastal sediments. 16S rRNA gene analyses confirmed dominance of *Gammaproteobacteria* in these sediments (Gihring et al., 2009). Phylogenetic analyses in the Arabian Sea confirmed that the library was represented by *Proteobacteria* (52%) (Divya et al., 2011), which confirms that *Proteobacteria* constitute an integral part of the soil metagenome at least on the west coast of India. How much of that contributes to the pathogenic bacteria can only be studied upon by using phylum specific primers and high throughput data collected from larger fragments of the variable region of 16S rRNA gene for a deeper analysis of the community structure.

Conclusions

The present study reports the dynamic nature of the benthic ecosystems of both the tropical estuaries, Mandovi and Zuari and demonstrates shift in the bacterial community structure influenced by the estuarine processes. Both the estuaries depict unique microbial groups, indicating different microbial activity which contributes to nutrient cycling in the sediments. Mangrove environments and the anthropogenic impacts seems to play a major role in structuring the communities and thus ecosystem functioning. Future studies should focus on the functional gene profiling of different microbial communities. It would be interesting to understand sediment-water linkages influenced by tides and seasons as this will help in pinpointing the health characteristics of the estuary.

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Legends to figures

Figure 1. Study area

Figure 2. (a) Metagenomic DNA along with 1kb ladder (M1) on 0.8% agarose gel; (b) PCR amplicons along with 100bp ladder (M2) on 2% agarose gel. Amplicon bands are coinciding with the corresponding 100bp band in M2 lane.

Figure 3. Domain wise distribution of sediment samples from Mandovi (Ma) and Zuari (Zu) estuaries.

Figure 4. Phylum wise distribution of sediment samples from Mandovi (Ma) and Zuari (Zu) estuaries.

Figure 5. Class wise distribution of sediment samples from Mandovi (Ma) and Zuari (Zu) estuaries.

Figure 6. Cluster Analysis with SIMPROF test of community structure classified into (a) Domains; (b) Phyla; (c) Classes of Zu-Ch [Chicalim], Zu-Si [Siridao] (Soil metagenomes of Zuari estuary) and Ma-Ca [Campal], Ma-Pa [Panjim] (Soil metagenomes of Mandovi estuary).

Legends to table

Table 1: Table showing the total bases, $\geq Q20$ bases, reads and mean bead length of all the samples. Zu-Ch [Chicalim]; Zu-Si [Siridao]; Ma-Ca [Campal]; Ma-Pa [Panjim].

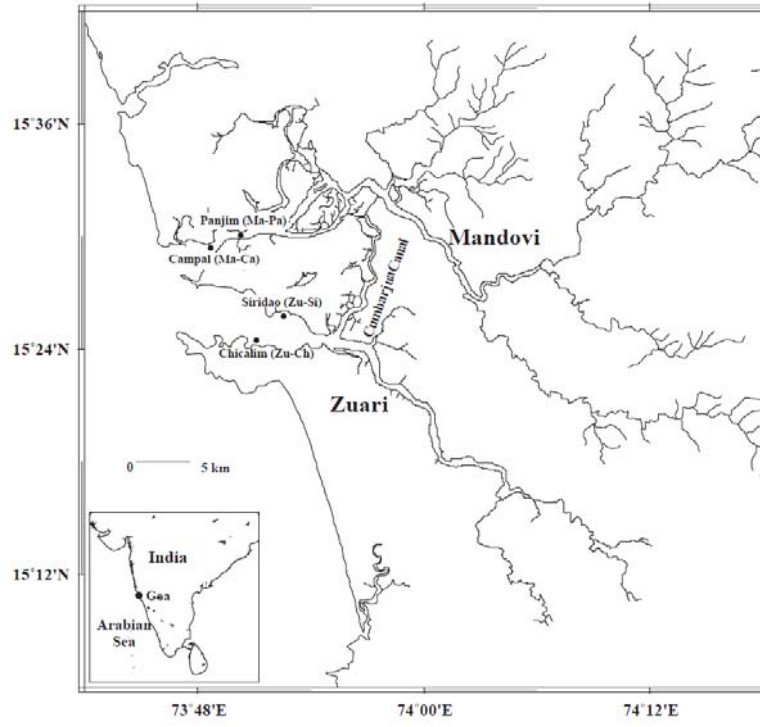


Fig. 1

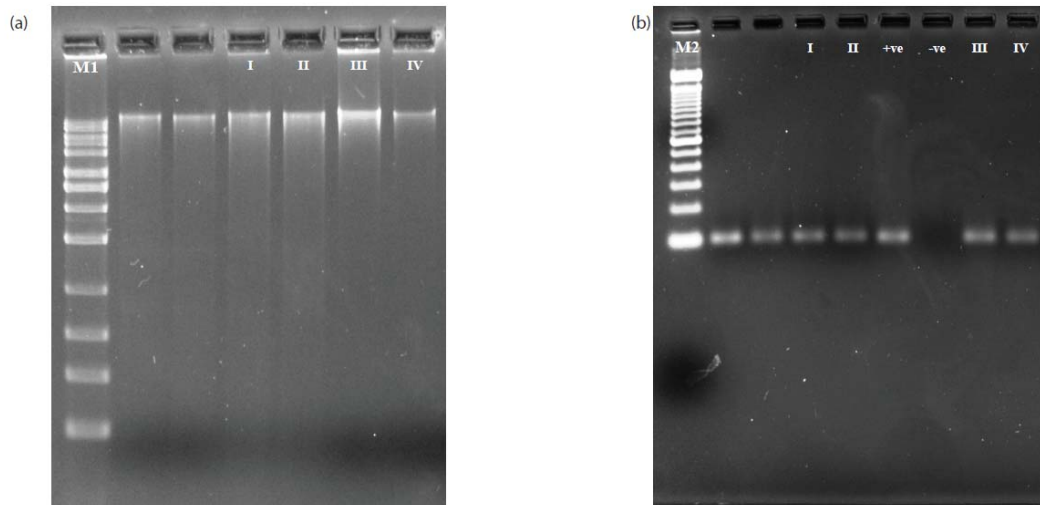


Fig. 2

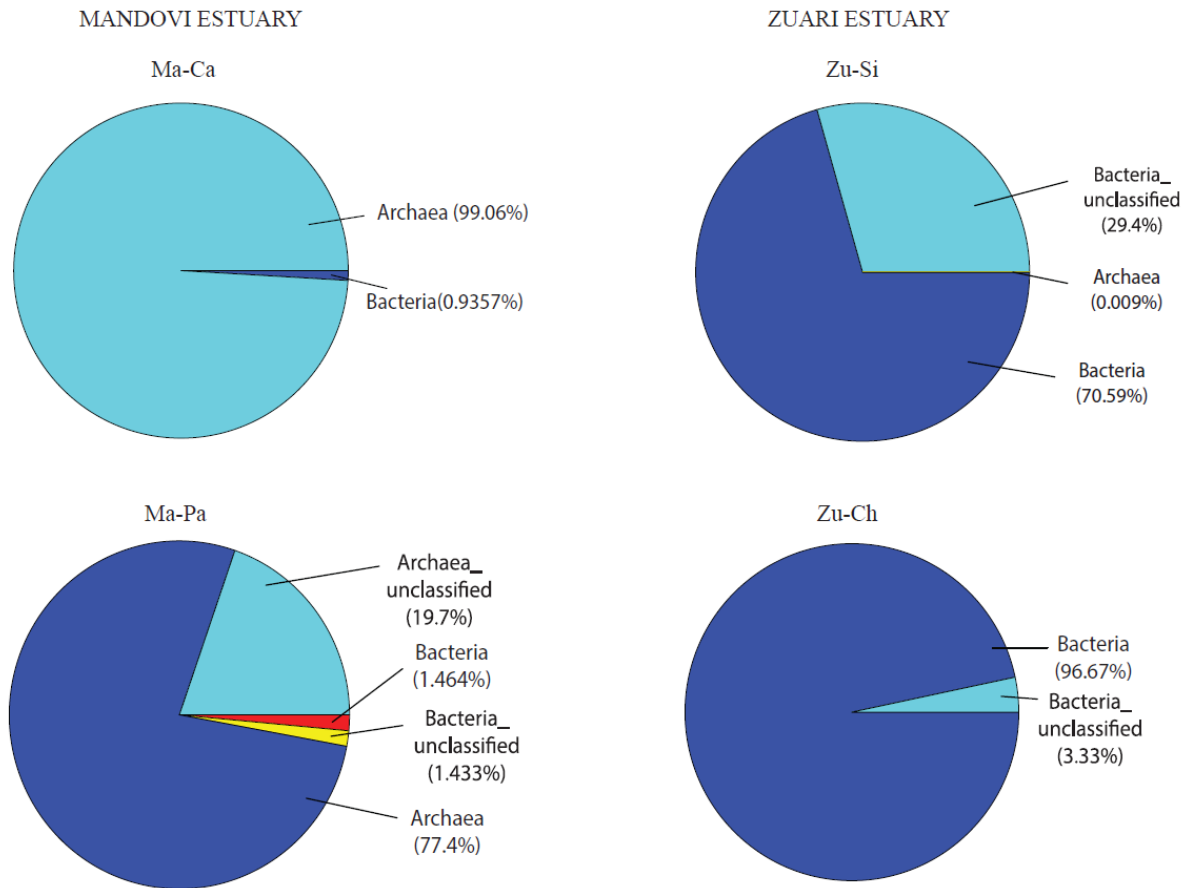
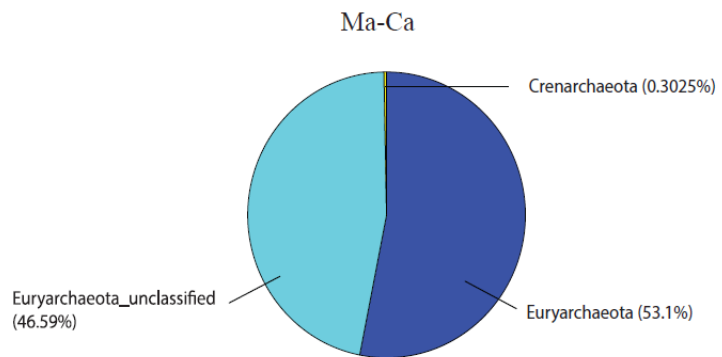


Fig. 3

MANDOVI ESTUARY



ZUARI ESTUARY

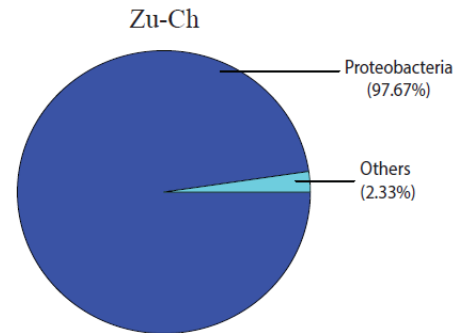
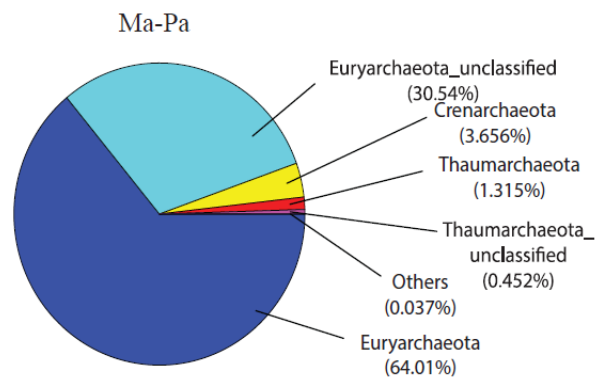
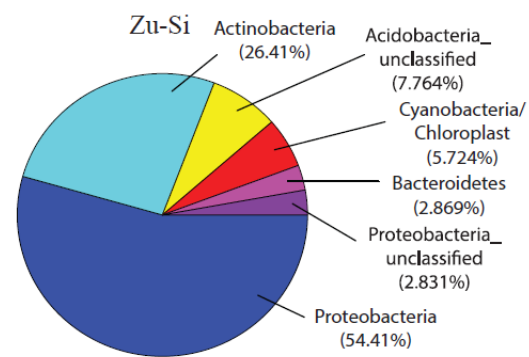


Fig. 4

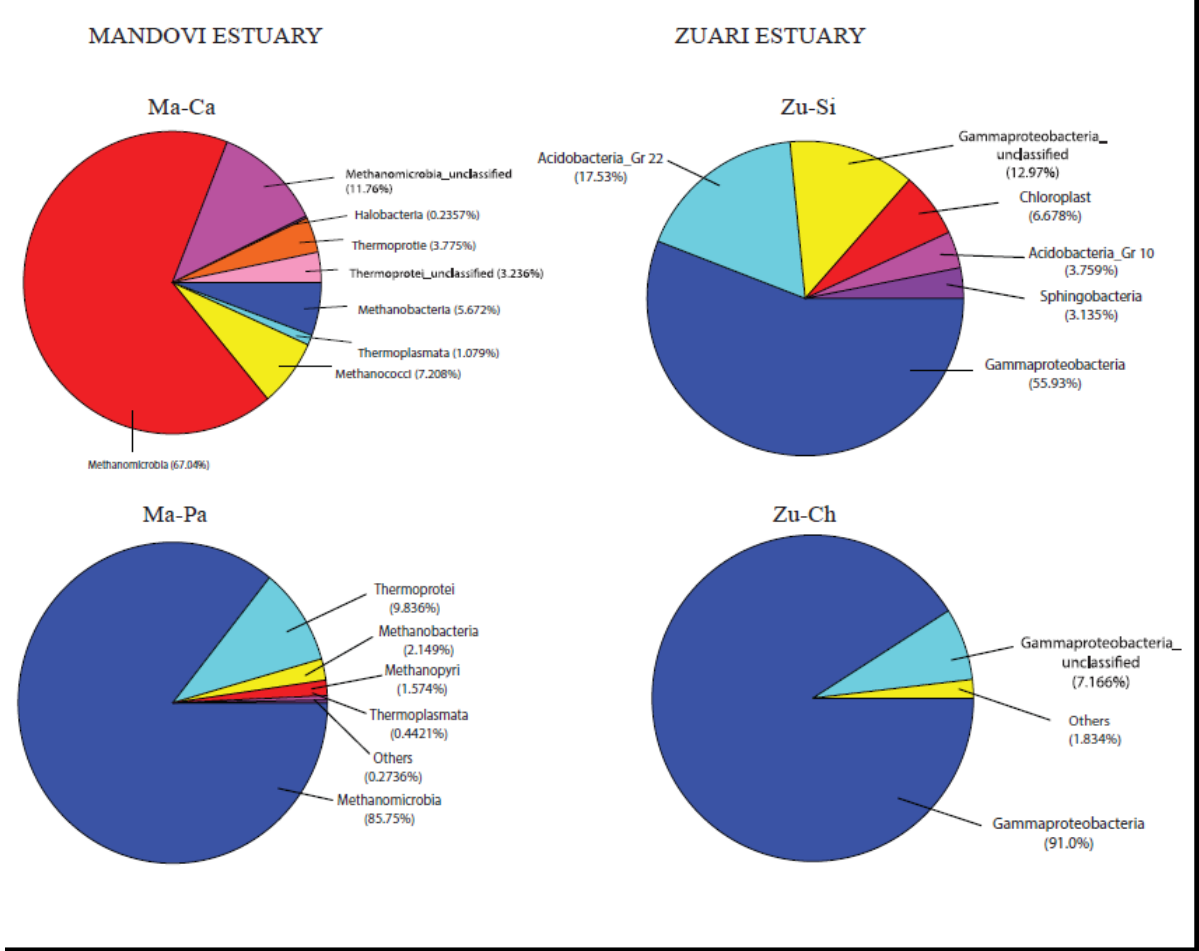


Fig. 5

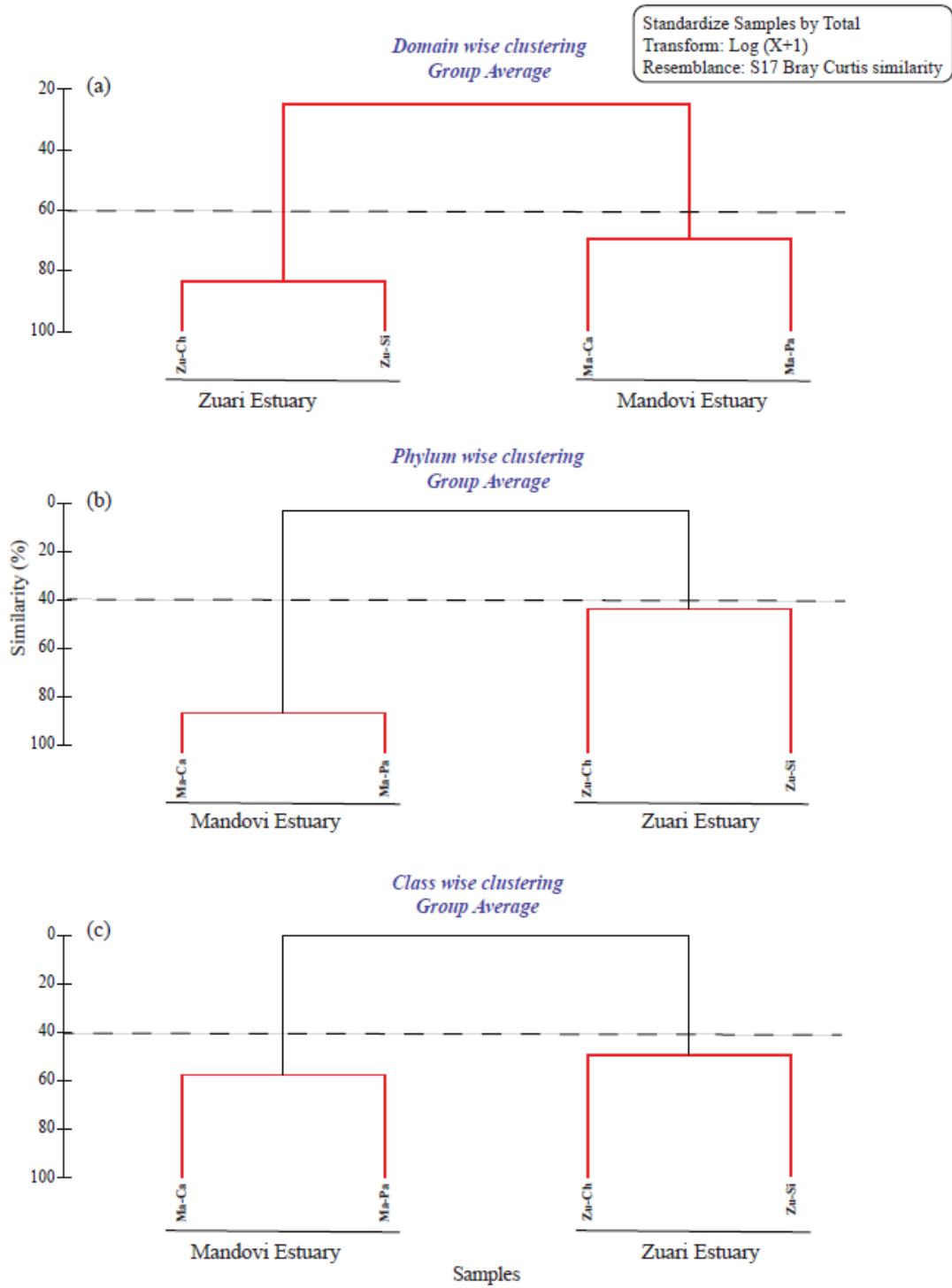


Fig. 6

Table 1

Barcode Name	Sample	Bases	=Q20	Reads	Mean Bead Length
IonXpress_002	Zu-Ch	1,952,376	1,737,926	21,295	91 bp
IonXpress_003	Zu-Si	2,522,295	2,256,641	27,363	92 bp
IonXpress_009	Ma-Ca	1,081,311	691,186	17,353	62 bp
IonXpress_010	Ma-Pa	1,008,150	705,019	14,271	70 bp