5. Discussion

In marine environments, microbes play an important role in sustainable maintenance of ecosystem by cycling nutrients through various biogeochemical cycles. They are the key mediators in maintaining the global biogeochemical cycling of essential elements like nitrogen, carbon, sulfur and methane. Studying microbial communities helps us to understand how microbes play essential roles in biogeochemical cycling and ecosystem functioning (Falkowski et al., 2008). Recent developments in molecular microbial ecology help us to understand the diversity and abundance of microbes and their function in an ecosystem. Although molecular approaches for assessing the microbial diversity in natural environments have been well developed and widely used in various types of environments (Urakawa et al., 1999; Iwamoto et al., 2000; Araya et al., 2003; Burgmann et al., 2004; Sousa et al., 2006; Muckian et al., 2007), our knowledge on the microbiology of mangrove ecosystems is still largely based on cultivation-dependent studies (Holguin et al., 2001). A few studies previously have addressed the microbial diversity in mangrove ecosystem, most of them focussing on novel taxonomical groups that inhabit this ecosystem. The major problem in cultivation based analysis is that only a small proportion of microbial population can be retrieved from any sample and rest remain undetected or uncultured (Amann, 1995; Vartoukian et al., 2010). To overcome this limitation many PCR based molecular studies like DGGE (Muyzer et al., 1993; Kowalchuk et al., 1997; Hasan et al., 2010), TGGE (Fouratt et al., 2003), SSCP (Backman et al., 2003, Bharatkumar et al., 2008) T-RFLP (Blackwood et al., 2007; Abramovich et al., 2012) and 454-pyrosequencing (Egge et al., 2013) have been invented which gives substantial information on the group of microbes that are present in this ecosystem.
Most of the studies on diversity focus on marine bacterial and archaeal distribution (Bhattacharyya et al., 2015), whereas little is known about the diversity of microbial group in mangrove rhizosphere. Mangrove ecosystem acts as a source of greenhouse gas mitigation and emission where the methane, carbon and nitrogen cycle were found to be predominant in this ecosystem (Bassi et al., 2014).

Here, the present study partially fills this gap, providing not only description of the bacterial and archaeal diversity, but also the diversity of key genes involved in nitrogen fixation and denitrification, their distribution in culturable and unculturables by comparing the DGGE profiles of the total bacterial communities associated with the rhizospheres of four different mangrove plants A. marina; R. mucronata; S. marina and S. brachiata. Moreover, the occurrence and diversity of the unculturable ammonia oxidizers were inferred by analysing the 16S rDNA of ammonia oxidizing bacteria and archaeal amoA, involved in nitrogen cycling process (Caffrey et al., 2007; Santoro et al., 2008; Cao Huiluo et al., 2011a,b; Wang et al., 2015).

**DGGE analysis of total bacteria and archaea:**

Although PCR DGGE has been used to analyse the microbial diversity the technique is associated with number of biases like DNA extraction efficiency, PCR primer annealing efficiency (Ranjard et al., 2000; Mammedov et al., 2008) and other inhibitory substances. It is very well known that the soils of mangroves are rich in humic acids (Sengupta and Chaudri, 1991; Holgiun et al., 1992; Vazquez et al., 2000) and humic acid contamination in the DNA would interfere with efficient PCR amplification. The humic acid were efficiently removed from the soil DNA were purified using MO-BIO soil DNA purification kit which yielded good quality DNA (Dineen et al., 2010). DGGE fingerprinting analysis served as
an appropriate molecular tool for culture-independent approach in rapid
detection of the predominant microbial community and enabled the
simultaneous analysis of multiple samples. The banding patterns and
numbers among all the samples indicated a diverse bacterial assemblage in
mangrove rhizosphere. DGGE analysis showed that diversity of the
bacterial community was rich compared to the archaeal community. DGGE
ribotyping provided information on different bacterial population
associated with different rhizosphere region of mangroves. In this study
the total bacterial diversity explored through nested PCR approach yielded
a better result than the direct PCR based amplification. From our study we
found that the diversity of bacterial population was more complex when
compared to archaea bacterial population. Previous studies have shown
that bacterial groups that belonged to phylum Proteobacteria,
Bacteroidetes, Actinobacteria, Acidobacteria, Firmicutes, Chloroflexi,
Spirochaetes, Cyanobacteria, and unclassified bacteria were common in
southeastern Brazilian mangroves (Gomes et al., 2010; Santos et al., 2011;
Silveira et al., 2011) and in other tropical and subtropical countries, such as
India, China, Australia, and the United States (Zhang et al., 2008), both
aquatic and sedimentary communities (Clementino et al., 2008; Vieira et al.,
2008). Our results also corroborated with the above findings such as
phylum Proteobacteria, Bacteroidetes, Chloroflexi, and Firmicutes were
found to be present in mangrove rhizosphere also. The presence of phyla
Ignavibacteria in this ecosystem is the first report on the presence of this
group in mangrove rhizosphere soils. In this study, based on RDP-classifier
analysis, all the phyla were classified up to family level. A total of 32
different families were identified of which 16 families belonged to
Proteobacterial group and 16 families belonged to other bacterial groups
like Bacteroidetes, Firmicutes, Chloroflexi, Actinobacteria and
Ignavibacteria. Previous studies have shown that Proteobacterial group
were dominant in marine ecosystem (Zhao and Zeng, 2008), similarly the prevalence of the Proteobacteria communities in Brazil mangrove soils and in grassland soils (Brons and van Elsas, 2008) were reported, our results have also shown that Proteobacterial group was found to be dominant in the Pichavaram mangroves. The distribution pattern was found to be similar across the mangrove rhizosphere regions. Within the Proteobacteria majority of the groups belonged to gamma proteobacteria followed by alpha, beta and epsilon proteobacteria. Some unclassified proteobacteria were also found in the sample. Our studies also showed that 46% of the total microbial life comprised of proteobacterial group followed by 25% Bacteroidetes group, 11% Firmicutes and 6% Actinobacteria, Ignavibacteriae and uncultured bacterial group. Dominant ribotype that was found to be present in rhizosphere region were found to be *Pseudomonas, Vibrio, Bacillus, Virgibacillus* and *Halomonas* species respectively. It has to be noted that gamma proteobacteria were found to be the dominant group present in the rhizosphere region. Two of the DGGE ribotypes AM30 and RM7 belonged to family Ectothiorhodospiraceae of gamma proteobacteria and was assigned to genus *Thioalkalispira* and *Thioprofundum* species by RDP-Classifier analysis. Both of these belonged to purple sulfur bacterium known to be present in salt lake and marine sediments (Sorokin and Kuenen, 2005). Sequences of DGGE ribotypes AM9, AM10, AM15 and RM15 classified into family Rhodospirillaceae, Rhodocyclaceae, Rhodobacteraceae and Rhodothermaceae were known to have metabolically diverse organisms capable of oxidizing sulfur and were known to be present in freshwater and marine environments (Melcher et al., 2002; Brakstad and Lodeng, 2005; Cytryn et al., 2005). The presence of these groups revealed that mangrove acts as source for all kinds of organisms performing various functions.
Very few studies have reported the archaea bacterial diversity in mangroves (Bhattacharyya et al., 2015). Due to the locations of the mangroves at the transitions from land to sea where there is a continuous inundation of river flow and the influence of tides, archaea bacteria are believed to adapt to these ever changing conditions. In this study almost all the samples collected from the rhizosphere of four different plants were found to harbor similar group of archaeal diversity except for the presence of one or two bands specific to particular plant species. The results obtained by Steven et al. (2008) from the Canadian High Arctic, showed that the 16S rRNA gene sequences belonging to the Crenarchaeota dominated the active layer and permafrost top layer region, while Euryarchaeota were predominant in the permafrost. However, Wilhelm et al. (2011) reported that both active layer and permafrost consisted predominantly of Crenarchaeota and the active layer had a greater proportion of Euryarchaeota compared with permafrost (4%). The archaea bacterial communities in Chinese mangrove (Yan et al., 2006) showed that the major archaeal groups were found to be affiliated to the phyla Crenarchaeota and Euryarchaeota. Other studies carried out in Brazilian mangroves by Andreote et al. (2012) showed that the major phyla in archaeal group belong to Thaumarchaeota and Euryarchaeota. The results obtained in the present study partially corroborate with the above reports, with similar dominance of Crenarcheota and Euryarchaeota phyla. The phylum Crenarchaeota was further represented by family Desulfurococccaceae representing Thermosphaera, Thermodiscus, and Stetteria and Pyrodictiaceae and Sulfolobaceae comprising Pyrodictus and Stygiolobus. The other phyla Euryarchaeota comprised of Haloferaceae and Methanomassillicoccaceae family comprising of Halobacteria and Methanomasillicoccus. Other studies based on 454-pyrosequencing data analysis on archaea bacterial diversity in Sunderban mangrove sediments
carried out by Bhattacharyya et al (2015) from India showed that the majority of the archaea bacterial sequences were found to belong to Halobacteria group. Unfortunately, only limited studies have been performed targeting bacterial and archaeal diversity in the rhizosphere region of mangrove plants.

**Diversity of Culturable and Unculturable Diazotrophic and Denitrifying community from mangroves**

The mangrove rhizosphere is a highly stressful environment and little is known about the bacterial groups present and the functions performed by them in this ecosystem. The conditions that prevail in the mangrove rhizosphere might be favourable for nitrogen fixation and denitrification which allow the sustenance of a these communities in that habitat (Holguin et al., 1992, 2001; Sengupta and Chaudhuri 1991; Zumft, 1997.) A few studies have parallely analysed both diazotrophic and denitrifying communities in any type of environment (Mergel et al., 2001; Rosch et al., 2002). Several works have been carried out towards diversity analysis based on culture independent methods and culture dependent method (Holguin et al., 1992; Gomes et al., 2010b). Their results allow determining whether different mangroves harbour the same bacterial population and whether distinct bacterial taxa were present in these regions.

In this study populations of free living nitrogen fixing bacteria and heterotrophic denitrifying bacteria have been analyzed from the rhizosphere region of four different mangrove plants. For assessing the diversity of culturable and unculturables both culture dependent and independent approach has been used in this study.

Bacteria and archea are major drivers of the soil nitrogen cycle, including nitrogen fixation, nitrification, denitrification, ammonification, anaerobic ammonium oxidizing and dissimilatory nitrate reduction (Lindsay et al.,
In this context, mangroves have not been explored to the same extent as other environments. The conditions that prevail in the mangrove ecosystem is most suitable for microbes involved in nitrogen cycling process which provides substantial activity for diazotroph and denitrifying bacterial community in these habitats. Previous studies have shown higher nitrogen fixing (Zubrer et al., 1978; Sengupta et al., 1991; Holguín et al., 1997; 2002) and denitrifying (Flores et al., 2007) activity in mangrove ecosystem. Hence, the selection of the mangrove as the study environment was not only due to its limited investigation but also due to the characteristics found in this ecosystem such as salinity and frequent anoxic conditions (Holguín et al., 2001; Ferreira et al., 2010).

### Unculturable and Culturable diversity of diazotrophs

*nifH* genes have been used as marker genes for studying the diazotrophic bacterial diversity. Bacteria harboring *nifH* genes have been reported in mangrove sediments, revealing the high activity of diazotrophic biodiversity in mangrove ecosystems (Zhang et al., 2008). In this study PCR-DGGE approach has been used to study the diversity of predominant unculturable nitrogen fixing bacteria by analyzing the diversity of the *nifH* gene from the extracted total soil DNA. Previous reports suggested that nitrogen fixing bacteria belonging to proteobacteria were dominant in marine ecosystem. *NifH* gene sequences affiliated with alpha, beta and gamma proteobacteria have been reported by Bagwell et al. (2002), from tropical seabed grass. Similarly Church et al. (2005 a,b) showed the presence of *nifH* like sequences identical to those previously described uncultured gamma proteobacterial sequences in North Pacific Ocean. The findings of Bird et al. (2005) suggested that gamma proteobacteria are predominant and acts as an important component of the heterotrophic nitrogen fixing microbial community of the tropical and subtropical
oceans. In this study, most sequences of nifH DGGE bands were similar to uncultured nitrogen fixing bacterial organism. Most of the nifH sequences showed a wide range of similarity from other environmental sources like high and low saline soil (Yousuf et al., 2014), marine sediments (Dang Hy et al., 2013), red sea (Foster et al., 2009), Mediterranean sea (Man-Aharonovich et al., 2007), rhizosphere of smooth cordgrass (Lovell et al., 2008), salt marsh (Lovell et al., 2012), fresh water, mangrove roots, agricultural and waste water treatment plants. None of the 29 nifH sequences in this study were clearly related to any known nifH clones of cultured nitrogen-fixing bacteria from mangrove ecosystems, not only from mangrove ecosystems but also from other sources, indicating the abundance of uncultured nitrogen-fixing bacteria in the mangrove rhizosphere soil. Mangrove ecosystem often contains nitrogen fixing cyanobacterial group (Kyaruzi et al., 2003), but in this study we weren’t able to see any of the bands correlating to cyanobacterial genes. Possibilities of detecting cyanobacterial nitrogen fixing genes by DGGE are less because of the intensity of the bands excised and sequenced in this study exceeded 1%. Another possibilities might be due to the primer set that has been adopted in this study. In the present study only one set of primers that targeted the nifH gene of bacterial community was used or the diversity of cyanobacterial population could be low in this ecosystem. But the primer set what we have used has given a good resolution and clear bands in DGGE analysis and has been proved to present a valid picture of the specific community the majority of the nitrogen fixing bacteria present in environment samples (Rosado et al., 1998; Piceno et al., 1999; Lovell et al. 2000; Piceno and Lovell 2000a, b; Bagwell et al., 2002). Our results suggested that combination of PCR based DGGE analysis of nifH gene along with gene sequencing and phylogenetic analysis served as a useful tool for investigating the
community profiling and identifying the diversity of predominant nitrogen-fixing bacteria of mangroves.

It is known that mangrove ecosystem are deficit in nitrogen content. Many research studies on diazotrophic activity in salt marsh ecosystems are closely associated with plant roots (Bagwell et al., 1998; Lovell et al., 2001; Larocque et al., 2004; Rameshkumar et al., 2008; Rameshkumar and Nair, 2009) The bacterial isolates were subjected to *nifH*-specific PCR amplification using two primers as described (Ueda et al., 1995; Poly et al., 2001), a rapid molecular method used to detect a wide range of diazotrophs and has been used as a functional gene to characterize diazotrophic communities in various habitats (Ueda et al., 1995; Poly et al., 2001; Zehr et al., 2003). The culture dependent studies basically depended on isolation and characterization of pure culture by 16S rDNA sequence analysis which assigned the organism a taxonomic position. In this study the diversity of the diazotrophs was determined by BOX-PCR fingerprinting along with polyphasic taxonomical identification of the positive isolates.

Nitrogen fixation in sediments is likely to be limited by insufficient energy sources whereas the mangrove rhizosphere sustained high rate of nitrogen fixing activity (Rameshkumar et al., 2008). The high diazotrophic activity associated with mangrove roots is probably due to root exudates, which are major sources of carbon and energy to the microflora immediately surrounding active plant roots. The same primers that has been used for DGGE analysis has been used to determine the diversity of culturable nitrogen fixing bacterial community. The 52 nitrogen fixing strains harbouring *nifH* genes that showed exact amplicon size of 360 bp when subjected to BOX-PCR fingerprinting revealed great variability among the positive isolates of nitrogen fixers. Advantages of using BOX-PCR based
analysis is widely recognised and is one of the most common tool for determining the diversity between closely related groups (Rademaker and Bruijn, 1997; Abd-ElHaleem et al., 2002; Marques et al., 2008; Ishii and Sadowsky, 2009; Ikeda et al., 2013). Previous studies suggested that the distribution of diazotrophs were more predominant in marine ecosystem and recently many members of nitrogen fixing bacteria belonging to proteobacteria have been studied from marine ecosystems. Bagwell et al. (2002) reported unculturable nifH gene sequences obtained from oligotrophic tropical seagrass beds showed similarity with the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria from the same ecosystem. Similarly Church et al. (2005) detected nifH gene sequences from North Pacific Ocean showed similarity to those previously described for an uncultivated gammaproteobacterial lineage. Bird et al. (2005) suggested that gammaproteobacterial nitrogen fixers are more predominant and plays a major role among heterotrophic diazotrophic community of tropical and subtropical oceans. Similar results obtained in our study showed that majority of the culturable nitrogen fixers belonged to Gamma proteobacterial group. High rates of nitrogen fixation were detected in association with dead and decomposing leaves, sediments, rhizosphere region, pneumatophores, and in subsurface sediments because of the ability of these microbial groups in colonizing terrestrial as well as marine sediments (Holguin et al., 1992; Mann and Steinke, 1992). BOX-PCR analysis showed that there are 24 different distinct clusters formed which showed a wide range of distribution among the positive isolates. This reflected a high degree of genetic diversity among the 52 PCR positive nifH strains isolated from different mangrove rhizosphere. Although, several culture dependent and independent studies on the occurrence and distribution of diazotrophs has been reported from various ecological niches only limited studies were from salt marsh ecosystems especially
from *Spartina alterniflora*, *Juncus roemerianus*, and *Salicornia virginica* (Bagwell *et al*., 1998; Bagwell and Lovell, 2000; Lovell *et al*., 2000; 2001; Larocque *et al*., 2004). This actually illustrates the abundance of uncharacterized diazotrophic bacterial species associated with different mangrove plant rhizosphere. The nitrogen fixers found in our study are similar to other reports from the mangrove ecosystem. For example, 5 isolates of nitrogen fixers isolated in this study belonged to the genus *Mangrovibacter*, confirming the members of same nitrogen fixing genera were isolated in mangrove rhizosphere (Rameshkumar *et al*., 2010). Previous studies by Liu *et al*., (2012) showed that 55.6% of the nitrogen fixing bacterial community belonged to gammaproteobacteria which indicated the dominance of this microbial group in mangrove ecosystem. Flores-Mireles *et al*., (2007) showed the distribution of microbial groups belonged to various genera such as *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium*, *Klebsiella*, *Vibrio*, *Phyllobacterium*, *Oceanimonas*, *Paracoccus*, *Corynebacterium*, *Arthrobacter*, *Aeromonas*, and *Pseudomonas*, isolated from the rhizosphere of mangrove ecosystems, while the results obtained in this study showed the distribution of nine different genera belonging to *Vibrio*, *Bacillus*, *Mangrovibacter*, *Klebsiella*, *Staphylococcus*, *Catenococcus*, *Serratia*, *Azospirillum* and *Rhodobacter*. It has to be noted that the results obtained by Flores-Mireles *et al*., (2007) are being corroborated with the results in this study except bacteria like *Mangrovibacter* and *Rhodobacter* has been reported in this study. Previous studies from our lab showed the presence of these *Vibrio* and *Mangrovibacter* (Rameshkumar *et al*., 2008; 2010) groups in this ecosystem in which these two microbial groups were found to be dominant. It is interesting to see that majority of the nitrogen fixers belonged to the genus *Vibrio* followed by *Bacillus*, *Klebsiella*, *Mangrovibacter*, *Serratia*, *Catenococcus*, *Rhodobacter* and *Azospirillum*. Nearly 69% of the isolates belonged to Gammaproteobacteria, 29% belonged to Firmicutes
and 2% belonged to Alpha proteobacteria. Our findings also clearly revealed Enterobactericeae (Mangrovibacter, Klebsiella and Serratia) along with Vibrio, Bacillus, Rhodobacter, Staphylococcus, Catenococcus and Azospirillum were abundant among diazotrophs. The nitrogen fixing bacterial group in Vibrio comprised of species such as V. plantisponsor, V. diabolicus, V. alginolyticus, V. parahemolyticus, V. natrigenes and V. neocladonicus. There is a considerable variation in BOX-PCR profiling of V. plantisponsor, V. alginolyticus and V. neocledonicus which was supported by phylogenetic analysis of these strains which formed an outward clade with the type strains. From Ez-Taxon analysis it is understood that the strains of species V. alginolyticus and V. neoclaedonicus cannot be distinguished based on 16S rDNA analysis and the difference in BOX profile of these strains suggest that these may be a novel species in this group. Similarly so far only two Mangrovibacter species has been reported (Rameshkumar, 2010; Zhang et al., 2015). Further the five isolates of Mangrovibacter species showed difference in BOX-PCR profiling from the reported strains indicating they can be novel species. The genus Bacillus obtained in this study showed similarity to B. aerophilus which has been previously reported in stratosphere region of earth’s atmosphere by Shivaji et al. (2009). Three isolates belonging to this species were isolated and BOX-PCR profile suggested that these may be novel species as considerable variation within the BOX profile was observed. It has to be noted that the strains B. aerophilus, B. startosphericus and B. altitudinis cannot be differentiated by 16S rDNA analysis. The results obtained in our study also suggested the same and hence further experiments have to be carried out to prove that these may be a novel species with diazotrophic activity.

The nifH gene of the culturable isolates were similar to uncultured nifH gene sequence and DGGE analysis also revealed the distribution of
uncultured nifH gene sequence which shows that majority of this sequence is yet to be assigned in taxonomic level. While the strains that belonged to Klebsiella showed similarity to Klebsiella nifH gene, Azospirillum showed similarity to nifH gene of A. brasilense. None of these genes were present in DGGE analysis of nifH gene. The reason might be due to the low distribution of these microbes in this ecosystem. This indicated a high degree of diversity of the nifH gene in the mangrove rhizosphere diazotrophic community. This result was consistent with Zehr et al. (2003), which showed more divergent regions in the nifH gene and differences in the rates of evolution between 16S rDNA and nifH genes. In this study, nifH sequences with more than 89% similarity belonged to the same genera, possibly indicating that nitrogen-fixing bacteria in the same genera have nifH sequences that are closely related to each other. According to this hypothesis, the nifH sequences from uncultured nitrogen fixing bacteria, which were 90–100% similar to the cultured bacteria (Vibrio, Mangrovibacter, Catenococcus, Bacillus, Staphylococcus, Serratia and Rhodobacter sp.) were identified as members of these genera. Most nifH sequences obtained in this study by the DGGE analysis shared 82–94% identity to uncultured nitrogen-fixing bacteria. As more nifH sequences were identified from cultured nitrogen-fixing bacteria, the uncultured bacteria might be assigned to defined genera.

These findings enhanced the knowledge on nitrogen-fixing bacterial diversity in mangrove ecosystems, supporting the occurrence of phylogenetically diverse novel lineages of nitrogen-fixing groups of prokaryotic organisms in the mangrove rhizosphere. DGGE along with the culture dependent technique showed a wide range of unexplored nitrogen fixing bacterial diversity in this ecosystem.
Unculturable and Culturable diversity of denitrifying bacteria:

A number of studies up to date have explored denitrifier communities from marine habitats but they all were from distinct geographic locations. A number of taxonomically unrelated bacterial groups capable of carrying out denitrification in marine ecosystems have been reported. Of these, 96% of cultured denitrifiers belong to the gammaproteobacteria (Braker et al., 2001), most of them belonged to the well-known genus Pseudomonas sp. Recently culture-independent approaches have been developed to analyse the diversity of denitrifying genes like narG, nirK, nirS, norB, norC, and nosZ (Braker et al., 2000; Prieme et al., 2002; Liu et al., 2003). The nosZ gene, encoding N₂O reductase, an enzyme catalyzing the final step of denitrification, is largely unique to denitrifying bacteria which is absent in most of the organism (Scala and Kerkhof, 1999). It represents the process leading to the loss of biologically available N from the sediment (Mills et al., 2008) and has been used as a marker gene for determining the diversity of denitrifiers (Horn et al., 2006).

This study uses nirS and nosZ gene as a molecular marker to determine the distribution and diversity of these genes in both culturable and unculturable populations of mangrove rhizosphere. The results obtained in this study showed that majority of the nirS gene and nosZ gene obtained through DGGE analysis belong to uncultured denitrifying bacterial group which indicates the much diverse and yet to explore denitrifying bacterial communities in this ecosystem. Studies by Braker et al. (2000) and Nogales et al., (2002) showed that diversity of nirS gene was dominant when compared to nirK gene.

The nirS primer pair showed efficient amplification of the nirS and nosZ genes from the denitrifying populations, of the four different mangrove rhizospheres. But the nirK primer pair F1acu and R3cu, were less efficient
in amplification of the nirK gene. The abundance of nirS genes detected in this study is consistent with that of the other comparable study of denitrifier marker genes in estuarine sediment (Smith et al., 2007). Majority of the nirS sequences obtained in this study have shown similarity to the sequences of various sources such as land fill leachate, estuarine sediments, activated sludge, waste water treatment plant, salt marsh and agricultural soil. Most of the nirS sequences in this study had a close match with those originally detected in estuarine and marine sediments as well as sludge (Nogales et al., 2002; Tsuneda et al., 2005; Osaka et al., 2006; Ruiz-Rueda et al., 2007; Spain et al., 2007; Bulow et al., 2008), suggesting that mangrove has both tidal and urban characteristics. Likewise, Nogales et al. (2002) studied the diversity of the nirS and nirK in the estuarine sediments and showed that only nirS was significantly predominant when compared to nirK. However, study by Smith et al. (2007) showed the decrease in nirS gene abundance with distance down an estuary, with possible relation to the nitrate and ammonia gradient; with no significant difference in nirS abundance between different rhizosphere regions suggesting that different factors influence the structure of the denitrifier community. DGGE analysis of nitrous oxide reductase (nosZ) gene also yielded the same result like that of nirS gene in which majority of them belongs to uncultured denitrifying bacterial group from various environmental sources. As reported in other environmental studies of the functional genes in the denitrification pathway (Enwall et al., 2005), most of the dominant nirS and nosZ types in our study clustered with other environmental clones. This indicates a large diverse population of unknown denitrifying bacteria in mangrove ecosystem. Most of the nirS and nosZ sequences in this study had a close match with those originally detected in estuarine, marine and ocean sediments (Nogales et al., 2002; Tsuneda et al., 2005; Osaka et al., 2006; Ruiz-Rueda et al., 2007; Spain et al., 2007; Bulow et al., 2008) as well as sludge and
agricultural ecosystem (Hallin et al., 2007; Orlando et al., 2012; Yoshida et al., 2012), suggesting that mangrove has both tidal and urban characteristics. NirS and NosZ DGGE analysis resulted in a few resolved bands, and only a limited number of phylotypes could be assigned.

Phylogenetic analysis of nirS and nosZ genes were found to form different clusters with ocean, marine and estuarine sediments in one cluster and agricultural isolates in another cluster which supported the wide distribution and yet to explore unknown bacterial lineages in this ecosystem. The DGGE analysis of nosZ using different primer combination with nosZF-GC as a common primer with two reverse primer nosZ1622r and nosZ1773r showed variation in amplification. Primer combination of nosZF-GC with nosZ1622r failed to give proper resolution in DGGE analysis while the primer nosZFGC in combination with nosZ1773r showed clear resolution when compared to the previous primer. This indicated that the resolution of DGGE was insufficient for nosZ amplicon.

Along with the unculturables the exploration of the culturable diversity of these two genes in culturable heterotrophic bacterial isolates showed the prominent distribution of these in Gamma proteobacteria group. The results obtained in this study were in coincidence with the previous studies of marine sediments ( Bowman et al., 2005 Zhao and Zeng, 2008), which showed Gammaproteobacteria was the most abundant denitrifying population in mangroves. Fast growing Gammaproteobacteria dominated estuaries (Green, 2010) as they have a preference for elevated concentrations of nutrients (Pinhassi and Berman, 2003). Thus the Gammaproteobacteria form the most abundant denitrifying communities in marine sediments (Bhatt et al., 2005). Brettar et al. (2002) showed that about 96% of cultured denitrifiers belonged to the Gammaproteobacteria. The culturable and non-culturable denitrifiers from Tuvem and Divar
mostly belonged to the class Gammaproteobacteria (Fernandes et al., 2010, 2012, 2014). Our results also were concurrent to the earlier report with predominant denitrifying community belonging to Gamma proteobacteria which consists of *Pseudomonas* and *Halomonas* group as predominant denitrifiers.

In order to understand their functionality in an ecosystem, further cultivation strategies are also desirable for recovering organisms with the novel sequences. Most of the dominant nirS clones were related to known cultivated denitrifiers, which indicated the most dominant members of the nirS containing bacteria in the community might be culturable. We were able to successfully screen and characterize some of the aerobic culturable denitrifying bacterial population from this ecosystem. In culturable analysis of denitrifiers, it was observed that majority of the isolates were from gamma proteobacterial group which belonged to the genus *Pseudomonas* sp. respectively. Different group of bacterial genera like *Halomonas, Labrenzia, Paracoccus, Nitratireductor, Bacillus, Virgibacillus, Shewanella, Staphylococcus* were observed to contribute to denitrifying activity. Among the different bacterial genera very few were able to perform complete denitrification process and the rest showed incomplete denitrification. It has been observed that the genus *Pseudomonas, Labrenzia, Halomonas, Paracoccus, Virgibacillus* and *Shewanella* were found to harbor both nirS and nosZ gene whereas other genera like *Bacillus, Staphylococcus* and *Nitratireductor* contained only nirS gene. The absence of nosZ gene in these organisms showed the inability of these isolates to perform complete denitrification which proved that majority of the denitrifying bacterial reported so far were partial and it confirms that denitrifying process can be performed by a wide range of genera (Zhou et al., 2001; Meyer et al., 2008; Bange, 2008). All these isolates were able to grow in nitrate broth and were
found to reduce it to nitrite. The genus *Pseudomonas* comprised of wide range of diversity which consists of species such as *P. balearica*, *P. bauzanensis*, *P. xiamenensis*, *P. stutzeri*, *P. xanthomarina*. Of these, dominant species was found to be *Pseudomonas balearica* followed by *P. bauzanensis*, *P. xiamenensis*, *P. stutzeri* and *P. xanthomarina*. This is the first study to show the presence of *P. balearica*, *P. bauzanensis*, and *Labrenzia* sp. from mangrove ecosystem. Previously *Labrenzia aggregata* has been isolated from marine sediments and only four species has been described so far in this genus. Of these bacterial groups *P. balearica*, *P. stutzeri*, *L. aggregata* and *P. kondratievae* were found to be vigorous denitrifiers as they can convert nitrate into gaseous form of nitrogen within 24 hrs of incubation under aerobic conditions. There is a strong correlation between DGGE analysis of denitrifiers and culturable denitrifiers. In DGGE analysis some of the sequences of *nirS* showed similarity to *P. balearica* and *nosZ* gene showed similarity to *Halomonas nitroreducens* which has been observed in culture dependent studies also. These results suggest that the predominant denitrifier present in the rhizosphere region were *Pseudomonas* and *Halomonas* which with 80% of the denitrifiers belonged to *Pseudomonas* sp. and 16% belonged to *Halomonas* sp. respectively which was also supported by the presence of these genes in DGGE analysis.

Based on the previous results, it is not known if *nirK* has an inherently low diversity in natural habitats or if the restricted diversity of *nirK* sequences is due to characteristics of the soils and sediments studied (Prieme et al., 2002). The heme cd1 containing Nir (*nirS*) occurred in a majority of numerically dominant isolates from rhizosphere region (Coyne et al., 1989). Even in DGGE analysis out of 37 bands eluted in this study only 7 were found to show similarity to *nirK* gene of uncultured isolates and also only four strains were found to be positive for *nirK* gene which belonged to
*Pseudomonas oleovorans*. However, primer preference for more conserved *nir* genes and possible bias during PCR amplification (Suzuki and Giovannoni, 1996) may partially explain the low distribution of *nirK* diversity. Thus, the F1acu, R3cu for DGGE analysis and nirK1F-nirK5R primer pairs for culturable analysis used in this study did not show any homology to the recently published *nirK* sequence.

The results presented here provide baseline data about denitrifier communities in mangrove rhizosphere region. The analysis was found to be reproducible. The cd1-nirS was found to be predominant in both culturable and unculturable analysis. The primer pair nosZ661bf with nosZ1527r yielded better result for culturable denitrifiers whereas the primer pair nosZF-GC with nosZ1773r yielded better resolution when compared to the amplicon obtained through nosZ-FGC and nosZ1662r.

It has to be noted that in 16S rDNA analysis of total bacterial population, predominant groups were found to belong to *Pseudomonas, Bacillus, Halomonas, and Vibrio* which coincided with the culture dependent studies where these groups contributed to nitrogen fixation and denitrification. It was also interesting to note that two strains from Rhodobacteraceae family such as *Rhodobacter johrii* and *Labrenzia agrregata* involved in nitrogen fixation and denitrification process were not reported earlier from the mangrove ecosystem. Total microbial community analysis in combination with culture dependent studies revealed some of the known groups and unknown groups involved in nitrogen cycling process were identified in this study.

**DGGE analysis of ammonia oxidizing bacteria and archaea**

The diversity, abundance, and activity of beta AOB and AOA were evaluated to provide insights into the microbial mechanisms driving
nitrification in mangrove rhizosphere region. DGGE analysis of bacterial ammonia oxidizers particularly the beta proteobacterium, dominant group in ammonia oxidizing bacteria were chosen to study the distribution of these groups in mangrove rhizosphere. Moreover the amo gene of bacterial ammonia oxidizers could not be amplified. Only archaeal ammonia oxidizers were successfully amplified with the primer specific for archaeal ammonia oxidizing group. Only few studies have been performed and analyzed in mangrove ecosystem in which 16S rDNA specific for bacterial ammonia oxidizers have been used instead of targeting the gene (Mahmood et al., 2006; Zhang et al., 2011).

Low recovery of AOB associated sequences from soil environments using the bAMO primer set has been observed in other studies (Bruns et al., 1999; Kowalchuk et al., 2000a, b; Freitag and Prosser, 2004). In marine-dominated estuarine systems, beta AOB belonging to the *Nitrosospira* dominate, but also members of the *N. marina* and *Nitrosomonassp.* have been identified (Francis et al., 2003; Cebron et al., 2004; Bernhard et al., 2005; Freitag et al., 2006; Mosier and Francis, 2008; Sahan and Muyzer, 2008; Jin et al., 2011). Studies by Laanbroek et al. (2012) showed differences among the distribution of ammonia oxidizing beta proteobacterial in mangroves where *Nitrosomonas* was found to be the predominant bacterial community. In this study, DGGE analysis of ammonia oxidizing bacteria and archaea showed that more or less same kind of population is present in the rhizosphere region of mangroves. DGGE and sequence analysis of bacterial ammonia oxidizers showed the presence of *Nitrosomonas* as the predominant ammonia oxidizing bacterial group with two bands showing similarity to *Nitrosospira* in RDP-Classifier program. The results indicated that *Nitrosomonas* were dominant beta proteobacterial ammonia oxidizing
group present in this ecosystem which corroborates with the results obtained by Laanbroek et al. (2012) and Coci et al. (2005).

Likewise DGGE analysis of archaeal ammonia oxidizers showed that the predominant group of archaea amoA belonged to Crenarchaeote group indicating the predominance of this particular group in this ecosystem. Previous studies on distribution of ammonia oxidizing archaea showed that member of phyla Euryarchaeota were found to be predominant in marine sediments (Munson et al., 1997; Purdy et al., 2002; Parkes et al., 2007), but the results obtained in this study showed that Crenarchaeota group were found to be the most dominant group in mangroves. Previous studies showed that AOA are found to dominate low ammonia environments such as oxygen minimum zone in estuary (Santoro et al., 2011) and open ocean (Pitcher et al., 2011). The Archaeal amoA sequences shared 93–99% identity at the DNA level and, may represent the ubiquity of archaeal amoA group in the rhizosphere region of this ecosystem. Such close similarity of archaeal amoA sequences of this study with sequences from soil, sediment, coastal sand from very distant geographical region might indicate minimal genetic diversity among dominant estuarine ammonia oxidizers present in mangrove rhizosphere. Ammonia oxidizing archaea amoA gene had a higher diversity than that of beta-AOB in this study, which was consistent with the previous results in marine sediments (Leininger et al., 2006; Wuchter et al., 2006; Lam et al., 2007; Mincer et al., 2007; Beman et al., 2008; Jin et al., 2011).

In the present work, diversity, abundance, and distribution of total bacteria, archaea, key genes involved in nitrogen fixation, denitrification, their diversity in culturable and unculturable analysis of ammonia oxidizing bacteria and archaea has been carried out through PCR-DGGE analysis and traditional cultivation methods. It is interesting to see that the
bacterial communities that were represented in DGGE analysis were cultivated under laboratory conditions and also found to be the major contributors in nitrogen fixation and denitrification. It is well known that mangrove ecosystem are nitrogen deficit and it is also known that nitrogen fixation is more predominant in mangroves and is well balanced by these group of isolates that involve in nitrogen cycling process (Zhang et al., 2008; Wickramasinghe et al., 2009; Schleper and Nicol, 2010). Through PCR-DGGE analysis in combination with culture dependent technique we were able to give a baseline data on the presence of microbial groups that performs these functions in mangroves. In this study we were able to identify that bacterial population in this ecosystem was much more complex and also some of the novel lineages of bacteria involved in nitrogen fixation needs to be thoroughly studied. Diversity of nirS and nifH gene in both culturable and unculturable was found to be abundant which is well supported by DGGE and traditional cultivation approach. The present work provides interesting data and novel information on our understanding of diazotroph and denitrifying community diversity in the mangrove rhizosphere. This is a preliminary study on diversity and distribution of microbial population involved in nitrogen cycle and more over the data obtained in this study is based on one particular season. Seasonal variation and changes has to be monitored in future studies for in depth understanding on shifts and functional aspects of this microbial population in this ecosystem. Finally we would like to infer that mangroves serves as a potential study site to understand the function of microbial communities, their role in global biogeochemical cycle and more over the possibilities of identifying new bacterial genera with potential functions can be identified and it serves as a hub for harboring novel lineages of bacteria.