Estuarine water column is subjected to wide fluctuations in various physical (tides, salinity, temperature and turbidity) (Shish and Ducklow 1994, Wikner et al. 1999, Cunha et al. 2000) and chemical properties (DO, Nutreints, pH etc) (Thingstad and Billen 1994, Amon and Benner 1996, Covert and Moran 2001). This system is persistently exposed to anthropogenic activities that cause negative impacts on their biodiversity and habitat suitability. It is well documented in temperate and sub-tropical estuaries that bacterial communities are heterogeneous in their abundance, physiological activity and diversity over trophic gradient (Hewson and Fuhrman 2004, Henriques et al. 2006). However, the biogeography and activity of the microbial assemblages in tropical estuarine environment has not been well understood (Dolan 2005). Nitrogen is considered as one of the major limiting factor in coastal waters (Howarth 1988). Estuaries are nutrient filters of coastal waters and thereby play a significant role in regulating the nutrients export from land to sea and making the nitrogen dynamics significant particularly in the estuaries. Human activities have considerably increased the availability of nitrogen in the biosphere (Vitousek et al. 1997), this excess nitrogen can leach from soils and enter to the natural aquatic system (Galloway et al. 2003) and finally to estuarine and coastal systems.
Estuaries may also serve as a significant nitrogen sink, owing to biotic removal by assimilation, denitrification or by burial processes, and also act as a source of nitrogen via direct discharge or degradation of organic matter. The major nitrogen pool in estuaries is total nitrogen (mostly organic nitrogen) and available inorganic nitrogen in water. The data presented by Downes (1988) suggested that nitrifiers play a major role in the dynamics of nitrous oxide in fresh water lakes. The data presented by (Downes 1988, Kim and Craig 1990) suggested that nitrifiers play a major role in the dynamics of nitrous oxide in fresh water lakes and nitrification is the origin of some nitrous oxide in deep ocean water respectively. However, the role of nitrifiers in the production of nitrous oxide in the oceans has been over estimated (Yoshida1988). In estuarine and coastal environment, nitrification is often coupled to denitrification (Jenkins and Kemp 1984, Sebilo et al. 2006). Hooper et al. (1997) also showed that some of the nitrous oxide may be produced through oxidation of ammonium. Nonetheless, recent studies generally consider nitrification as a major player in nitrous oxide production (Seitzinger and Kroeze 1998, Punshon and Moore 2004, Meyer et al. 2008).

Nitrification and nitrifiers in estuaries is modulated by the complex interplay among different microorganisms and between microorganisms and environmental variables, which in turn is dictated by various hydrodynamic characteristics like fresh water discharge and seawater influx. The highly dynamic system of tropical estuaries is an appropriate platform to study the influence of environmental parameters on the growth and activity of nitrifiers. Despite its importance, the studies on nitrifiers in tropical estuaries have eluded researchers and this area has lagged behind its pelagic counterpart.

5.1 Estuarine Characteristics

During the study period, the CE showed the characteristic of a typical micro tidal tropical estuary. The major hydrological variable in the CE was salinity which varied from limnetic to 35. This is similar to the situation encountered in any other Indian estuary where the salinity gradually declines from ~35 at the mouth to ~0.1 at the entry point of the rivers (Qasim 2003). The CE is influenced by two main factors viz. the short term changes induced by the tides and the long term
seasonal changes brought about by the monsoon system. The tides at the CE are mixed and semidiurnal in nature. Two high (flood) and two low (ebb) watermarks occur every day and these differ in amplitude (Srinivas et al. 2003). Though it is reported that the seawater through tidal intrusion reaches up to 60 kms towards the head of the estuary (Balachandran et al. 2008), the observed low salinity was mainly due to fresh water discharge from the six rivers that open into the estuary. The influence of fresh water became more obvious during the monsoon season because 1) the annual rain fall during the monsoon season along the southwest coast is close to 70% of the total and this voluminous fresh water finds its outlet into the estuary through the rivers (Srinivas et al. 2003) and 2) the high river discharge limits the saltwater intrusion through the two permanent openings from the Arabian Sea to a short distance of only up to 25 kms from the mouth into the estuary (Balachandran et al. 2008). The effect of the fresh water input during the monsoon is carried up to the coastal station (Stn. 4 which is about 20 kms away from the mouth of the estuary) where the salinity dropped from 35 to 14. Post-monsoon season is a transitional period, when the river discharges gradually decrease, the tidal influx gains momentum and the estuarine conditions turns to a partially mixed type thereby weakening the stratification (low salinity prevailing at the surface waters). High salinity in the CE during the pre-monsoon season (dry and stable period) is due to the low river input at the upstream, resulting in extended seawater intrusion into the estuary. Thus in the CE, fresh water mostly regulates the salinity gradient than the sea water intrusion. The water column temperature ranged from 24 to 32° C in the CE showing the typical nature of a tropical system. Water column was relatively cool during monsoon period (27.9 ± 1.6) and intense solar radiation increased the temperature relatively high (30 ± 1.6) during pre-monsoon and post monsoon seasons (29.3 ± 0.8).

The concentration of DO ranged from 2.16 to 7.47 mg L⁻¹ in the CE. The oxygen concentration at the surface and near bottom water varied marginally between and within the stations. The average of observed DO in the water column (5.04 ± 1.4 mg L⁻¹) reflects healthy oxygenated water throughout the year except at few sampling occasions. This is mainly due to the mixing of water column by the tidal and fresh water influence. During few sampling occasions the DO decreased
drastically (down to 2.16 mg L$^{-1}$) may be because of stratification of the water column and the high oxygen consumption rates. Rates of microbial community respiration have been reported to decrease the DO concentrations below 0.8 mg L$^{-1}$ in Chesapeake Bay waters (Sampou and Kemp 1994).

In the CE, the levels of dissolved inorganic nitrogen were high throughout the study period. Such high levels have been earlier reported from the CE, and this has been assigned mainly to industrial effluents and domestic sewage (Qasim 2003, Madhu et al. 2007). The concentration of inorganic nitrogen in estuarine stations ranged from 5.42 to 61.54 μM (during pre-monsoon), from 8.72 to 32.41 μM (during monsoon) and 10.23 to 57.73 μM (during post-monsoon) seasons. However, the concentrations were comparatively low in the coastal region during all the seasons. In this study, the post-monsoon values showed an increase in ammonium and nitrate as compared to the monsoon, but they were still lower than the pre-monsoon values. During the non-monsoon seasons, the physical conditions favour physiological and regenerative process of ammonium production and, as water movement is low, there is accumulation of nutrients in the water. Since the concentration of the nutrients viz. ammonium, nitrite and nitrate were comparable it appears that the prevailing conditions in the estuary were conducive for nitrification. In addition, in the present study, there was no significant correlation between ammonia, nitrite and nitrate, suggesting that these species of nitrogen had different controlling mechanisms. The utilization of nitrite by the autotrophs is also an important factor that could influence the nitrite pool significantly. Further it was observed that significant correlation was seen between nitrate and nitrification only during the pre-monsoon period collaborated to the fact that in the non-monsoon months, nitrate concentrations are controlled by microbial activity (nitrification) whereas, in the monsoon season it may have been influenced by transportation from watersheds. The dissolved inorganic nitrogen content observed in the CE was much higher than the reported values from other estuaries of India (Ram et al. 2003, Sarma et al. 2010, Sarma and Rao 2013). However high nutrient loading during the monsoon season has been earlier documented not only in the CE but also in other tropical estuaries such as the Mandovi-Zuari (Ram et al. 2003), Godavari (Sarma et al. 2009, Sarma et al. 2010), and Hoogly (Mukhopadhyay et al. 2006) around the
Indian peninsula. The nutrient concentration observed in the CE was comparable with that of major world estuaries like the Seine estuary in France (Garnier et al. 2006), Schelde estuary in Belgium (De Bie et al. 2002), and California estuary in USA (Boyle et al. 2004). Higher levels of N/P ratio was observed during pre-monsoon (av. 95.52 ± 120.72) and post-monsoon (av.37.16±26.6), which is significantly above the Redfield stoichiometry (16:1). Our observation corroborates the earlier observation on nutrient overloading in the CE, leading to eutrophication, which may further intensify in future if not regulate (Martin et al. 2011). Maximum terrestrial inputs enter in to the estuary during first onset of monsoon and it gradually reduces towards the peak monsoon season. Heavy rainfall and associated high fresh water influx for 4 to 5 months washes up the system and thus minimize the nutrient accumulation in the CE.

High SPM  was observed during all the seasons (pre-monsoon average, 54.7 ± 53.5 mg L$^{-1}$, monsoon average 37.5 ± 36.4 mg L$^{-1}$ and post monsoon average, 55.9 ± 41.4 mg L$^{-1}$) normally indicates the turbid condition of the estuary. Large quantity of suspended materials in the CE throughout the study period may be in the form of detritus (large fallout of plant and animal material) and sediments (terrestrial and riverine origin and from re-suspended from the bottom). This is primarily attributed to both autochthonous and allochthonous materials during the non-monsoon periods and more of allochthonous materials during monsoon (Madhu et al. 2007). It is also reported that phytoplankton biomass can be the major part of the SPM during pre-monsoon period in the CE (Madhu et al. 2007). The large quantity of SPM observed in the resultant water column turbidity limits light penetration in the euphotic zone (Qasim and Sankaranarayanan 1972) and since the primary production is light limited in the estuaries (Alpine and Cloern 1992), it significantly influence the entire productivity of the estuary. It can be concluded that in the CE most of the water quality parameters (temperature, salinity, DO, pH, SPM, nutrients etc), are primarily influenced (distribution in the estuary and concentration) by the tides, but the temporal changes in the hydrology is controlled by the prevailing SW monsoon.
5.2 Distribution of Eubacteria and Archaea

The total prokaryotic count (DAPI count) ranged from 7.53 × 10^5 to 1.99 × 10^6 cell ml\(^{-1}\). This count represents the total prokaryotic load in the CE and was in agreement with earlier studies in Cochin estuary (Thottathil et al. 2008, Parvathi et al. 2014) and other Indian estuaries (Ram et al. 2007, Sarma et al. 2011). The population of Eubacteria and Archaea estimated based on FISH ranged from 3.3 to 6.91 × 10^5 and 1.93 to 5.48 × 10^5 cells ml\(^{-1}\) respectively. Eubacteria dominated the estuarine microbial community and accounted for 31 to 55% (average 46%) of the total prokaryotic population detected using DAPI counts, while Archaea accounted for 19 to 31% (average 27%). The planktonic Archaea in the CE, though make up for a small but significant percentage of the total microbial population. The eubacterial counts were comparable with other estuaries in the SW coast of India and also with other estuaries in the world. Whereas abundance of Archaea could not be compared with that of other Indian estuaries as the same is being reported for the first time, however it can compare with other world estuaries and coastal system. Quan et al. (2010) estimated the abundance of Eubacteria about 52 to 82% of total prokaryotic abundance in Daliao river water system and its estuary from NE China. However archaeal contribution (1 to 11.8%) in their study and was less than the present observation. Similar studies in two temperate estuaries namely Choptank and the Pocomoke River estuary (both sub-estuaries of the Chesapeake Bay) are also observed comparable results (Bouvier and del Giorgio 2002). In that studies Eubacteria enumerated was an average 32% of the total number of cells determined by DAPI direct counts and the probe targeting members of the Archaea detected on average less than 3% of DAPI counts along the estuaries. The archaeal abundance observed in this study was relatively higher than the previous reports from estuaries while it was in agreement with coastal and marine system as showing below.

The planktonic Archaea in the CE, though a small in number was a significant percentage of the total microbial population. Water column of the Eastern Mediterranean Sea also showed comparative results, where Eubacteria contributed between 24 and 72% to total prokaryotic abundance and Archaea
contribute up to 35% (La Ferla et al. 2010). But the contribution of bacteria to total prokaryotes did not differ significantly among the stations and did not show any depth related pattern (La Ferla et al. 2010). Dominance of bacteria over archaea was also evident in other marine environments. Ye et al. (2009) reported higher abundance of bacteria ranging from $5.8 \times 10^6$ to $3.3 \times 10^8$ gene copies g$^{-1}$ sediment than archaeal abundance from $4.3 \times 10^4$ to $8.5 \times 10^5$ gene copies g$^{-1}$ sediments using 16S RNA gene PCR method in the Mississippi Canyon. Teira et al. (2004) reported that in the deep waters of the North Atlantic Eubacteria were more abundant than Archaea (42% versus 32% of DAPI counts) and the percentage of bacteria decreased with depth, whereas archaeal abundance increased with depth (Teira et al. 2004). Similar results were also reported by Herndl et al. (2005) in Atlantic sea. Their study also indicated the importance of archaeal production in the deeper ocean and although its productivity is generally lesser than that of bacteria it can reach up to 84% of total prokaryotic production. However it has been suggested that Archaea are possibly outcompeted for resources by other microbial populations in less extreme environmental conditions, but they dominate in more extreme natural environments such as deep pelagic waters or cold Antarctic waters (Murray et al. 1998).

In this study it was found that depth did not have a significant effect on microbial abundances as surface and bottom waters did not show any significant variation in abundance of these two groups of microorganisms. This result is not in agreement with previous studies that reported archaeal densities increased with depth (Massana et al. 1997, Fuhrman and Ouverney 1998). This may be due to the mixing of water column and also could be because the study stations in the CE are shallow compare to the deeper waters. The spatio- temporal distribution of both groups showed similar pattern, with an increase in cell numbers during the pre-monsoon followed by a decrease during the monsoon season. So it can be suggested that Eubacteria and Archaea in the CE have similar response to the environmental factors that determining the microbial population. High water resident time during pre-monsoon and least water resident time in monsoon (Revichandran et al. 2012) may be the critical factor that regulated the microbial load in the CE. Based on the study in the turbidity maxima region of the Columbia estuary, Crump et al. (1999)
suggest that the free living bacteria may not develop into a uniquely adapted estuarine community due to a short residence time of water (during high rainfall period). However, during low rainfall periods the retention of water in the estuary was theoretically sufficient for development of a bacterial community.

As reported for other estuaries, (Bouvier and del Giorgio 2002, Kan et al. 2006), it is probable the environmental factors like residence time, salinity dissolved oxygen and nutrients composition and concentration are the major factors in determining the composition of microbial community in the CE. Inorganic and organic nutrient availability may profoundly influence not only microbial metabolism but its community composition (Rappé and Giovannoni 2003). Both Eubacteria and Archaea were found to be affected by water salinity (r = -0.49, p<001, n = 48; r = -0.31 p<001, n = 48, respectively) and cell numbers increased with decreasing salinity at all the stations. Maximum abundance of Eubacteria and Archaea were observed in the low saline station, and at the times of high nutrient concentration. However, the count was found decreasing during the time of peak monsoon. Such inverse significant correlation with salinity has been reported from other estuaries (Iriarte et al. 1997, Bouvier and del Giorgio 2002, Hewson and Fuhrman 2004). Nitrate concentration was the other factor that showed influence on eubacterial and archaeal distribution whereas ammonia concentration showed influence with archaeal abundance only. The distribution pattern of Eubacteria and Archaea in the CE and its response to varying environmental parameters suggests that salinity could be an important controlling factor, in addition to quantitative and qualitative changes in the nutrient composition. Sodium inhibition has previously been suggested to explain the higher occurrence of microbes in freshwater and low salinity coastal waters (Hiraishi et al. 1991, Rheinheimer 1997).

5.3 Distribution AOB and NOB

After the first reports on the successful isolation of chemo-lithoautotrophic ammonia oxidizers at the end of the 19th century (Winogradsky 1890), researchers continued to investigate the diversity of ammonium oxidizers in natural and engineered environments by applying enrichment and isolation techniques. However, low maximum growth rates and growth yields of ammonium...
oxidizers render cultivation based analysis of their environmental diversity extremely time consuming and tedious. Furthermore, all culture techniques are potentially selective and thus bear the risk of incomplete coverage of the actually existing bacterial diversity (Amann et al. 1995, Wagner et al. 1995, Theron and Cloete 2000). Recently, a battery of molecular tools to infer the presence of ammonium oxidizing bacteria in the environment has been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural gene \textit{amoA} (Holmes et al. 1995, Sinigalliano et al. 1995, Rotthauwe et al. 1997, Mendum et al. 1999). Quantitative information on ammonium oxidizing bacterial population structure and dynamics in the environment is obtainable via membrane or \textit{in situ} hybridization techniques in combination with ammonium oxidizing bacteria specific oligonucleotide probes (Wagner et al. 1995, Mobarry et al. 1996, Wagner et al. 1996, Schramm et al. 1997, Juretschko et al. 1998, Logemann et al. 1998). The latter approach also allows one to directly relate community structure with the morphology and spatial distribution of the detected organisms. On the other hand, more sensitive quantitative PCR techniques also have limitations in that it measures the copy number of genes and not the number of organisms. For example AOB having an average of 2.5 gene copy number of ammonia monoxygenase gene per organism will give a count of approximately double the number of actual AOB present (Norton et al. 2002). The observed function diversity is not well supported by the taxonomic diversity of nitrifiers hitherto recorded.

The range of abundance of nitrifiers in this study was $\beta$ AOB (3.15 to $9.3 \times 10^4$ cells ml$^{-1}$), \textit{N. mobilis} (1.01 to $4 \times 10^4$ cells ml$^{-1}$), \textit{Nitrobacter} sp. (2.69 to $7.63 \times 10^4$ cells ml$^{-1}$) and \textit{Nitrospira} sp. (2.51 to $6.17 \times 10^4$ cells ml$^{-1}$) in the water column they showed a spatial and temporal variation. Nitrifiers counts in the order of $10^4$ ml$^{-1}$ may signal the possibility of novel groups of nitrifiers (inference drawn based of limited cultivable nitrifiers) though the probes used in this study are known to cover most of the nitrifiers. A direct comparison of the FISH results with water column of world estuaries is difficult as majority of the reports on the distribution of nitrifiers are restricted mostly to sediment samples, enumerated using MPN or quantitative PCR technique (Dai et al. 2008, Mosier and Francis 2008, Jin et al. 2011). The available references on FISH based enumeration of nitrifiers are widely
reported in sewage systems (Wagner et al. 1996) and anammox in oxygen minimum zone (Schmid et al. 2007). The observed abundance of AOB in the CE is less than in Elbe Estuary where AOB population was at a magnitude of $10^3$ cells ml$^{-1}$ (Stehr et al. 1995). The abundance of nitrifiers in the Pearl River estuary in China, measured using MPN methods, was reported to range from 2 to $4 \times 10^3$ cells ml$^{-1}$ (Dai et al. 2008), which is approximately one order less than the nitrifiers counted in the CE. It can be presumed that this difference may be due to the limitations of culture dependent techniques, which permits the growth of only actively growing organisms. When looking to the qPCR based abundance estimation of nitrifiers, range of $10^4$ to $10^7$ copy numbers of β-AOB amoA gene have been reported from a millilitre of water sample from temperate environment (Laanbroek 2013) and estuarine sediment. For example Mosier and Francis (2008) has reported the abundance of β-AOB amoA ranging from $3.1 \times 10^4$ to $5.3 \times 10^7$ copies g$^{-1}$ in the sediment of San Fransisco Bay Estuary. If the limitations of both MPN and qPCR techniques are accommodated and extrapolating the numbers with reference to sediment, the observed the abundance of AOB and NOB in the CE is not under estimated.

Previous studies have reported that Nitrobacter sp. and Nitrospira sp. are the major nitrite oxidizing organisms in the nutrient rich estuaries (Cébron et al. 2003). Abundance of Nitrobacter sp. and Nitrospira sp. in the CE were in the order of $10^4$ and these results are in agreement with those of other estuaries such as the Sein river estuary, where Cébron et al. (2003) reported an abundance of from $9.8 \times 10^2$ to $3.6 \times 10^4$ Nitrobacter sp. cells ml$^{-1}$. Their study reported sharp decrease and increase in abundance of Nitrobacter sp. with changing salinity and other environmental parameters. Though there was a significant spatial and temporal variation in abundance of Nitrobacter sp. in the CE, such sharp changes in abundance was not observed and the abundance was in the order of $10^4$. The possible reason for this contrast is that NOB community in the tropical region may be more adaptable towards the environmental variations.

The distribution pattern and seasonality of AOB and NOB in the CE suggest the coexistence of these organisms, which are responsible for modulating
the entire nitrification process in the estuary. AOB and NOB showed similar response to important physical and chemical characteristics of the environment. It has been reported that the coexistence of AOB and NOB may create a suitable micro niche that support the growth and activity of each other (Costa et al. 2006). For instance, the nitrite released by AOB could be utilized by NOB. A direct measurement of the abundance of AOA was not done in the present study due to the technical limitations of FISH to measure low copy number functional genes. Results showed the possibility of a cross feeding between ammonia oxidizing microorganisms (AOB, anammox, and AOA) and NOB. The cross-feeding between AOB and NOB has been reported earlier in biofilms, using confocal microscopy and microautoradiography fluorescent in situ hybridization (MAR-FISH) techniques (Okabe et al. 2004).

In estuaries, the environmental factors co-vary depending on the seawater influx or freshwater discharge and hence it is not a single parameter but the co-influence of different conditions that would determine the distribution and activities of microorganisms. Salinity (Caffrey et al. 2007) ammonia, (Jones and Hood 1980, Dang et al. 2008, Cao et al. 2012) and temperature (Iriarte et al. 1997) are considered as the predominant environmental factors influencing nitrifiers in estuaries, while influence of SPM, pH and other dissolved nutrients have also been discussed in literature (Allison and Prosser 1993, Cébron et al. 2003). Allochthonous ammonia reaches the CE through various routes including domestic and industrial wastes and land runoff. In the CE, ammonia was the nutrient which had a significant influence on the distribution of both AOB and NOB (r = <2.8, p<0.01, n = 48). Ammonia forms the first substrate for initiating the rate limiting step of nitrification; therefore it can influence AOB as a substrate and NOB as a source of substrate and such relations are obvious in estuarine and marine environments (Bouskill et al. 2012, Cao et al. 2012). In agriculturally impacted Elkhorn Slough Estuary in California, high AOB amoA gene abundance was recorded when the concentration of organic content and ammonia are high (Wankel et al. 2011). Although there was marked difference in the abundance of AOB and NOB between seasons in the CE, salinity could not be established as the critical factor controlling their abundance. This indicates that the seasonal variation in
Abundance of AOB and NOB in the CE are being modulated by freshwater discharge rather than seawater influx. Further, reports from other estuaries suggest that intermediate salinity may be the preferred environment for nitrifiers (Rysgaard et al. 1999, Bernhard et al. 2007, Bernhard and Bollmann 2010). For example in the Plum Island Sound estuary, Bernhard et al. (2010) documented no linear relationship between AOB abundance and salinity, but observed that there was a pattern of high AOB abundance in intermediate salinity of ~ 20. Similarly pH also did not show much influence on the abundance of AOB and NOB in the CE, as the observed pH in the CE was near neutral or slight alkaline. The DO levels of the water column in the CE varied between seasons and was always well above the minimum concentration, i.e. 1 to 1.5 mg L\(^{-1}\) required for maintaining the growth and activity of both AOB and NOB (Garnier et al. 2007). Therefore no correlation was observed between the oxidizers and DO in the CE.

Estuaries not only act as a transition zone for fresh and marine waters but also for microorganisms from these two different environments, and in an active estuary these microorganisms may play a fundamental role in the ecological functioning of the system. The abundance and distribution of nitrifiers in the CE is controlled by a combined effect of river water discharge and flushing. Flushing activity would be inactive in the CE during pre-monsoon (Revichandran et al. 2012), while it experiences multiple flushing in addition to heavy rain fall during monsoon. In concurrence with this, the ammonia levels and abundance of AOB and NOB were found to be higher during pre-monsoon and lower during monsoon. In a seasonal perspective, in the CE the availability of ammonium is the most important factor governing the abundance of autotrophic nitrifiers during all the seasons. Interestingly, the CDA analysis showed that estuarine and coastal regions of the CE formed different clusters with respect to the abundance of nitrifiers (AOB and NOB) and environmental variables. It is clear that the estuarine region of the CE (Stns. 1-3) being more dynamic due to tidal influence and more anthropogenic activities than the coastal region (Stn. 4). Further, less stability was observed in the estuarine region as indicated by more factors influencing the abundance of AOB and NOB with less prediction efficiency (less VE) compared to coastal region.
5.4 Community Structure of AOB and AOA

Many Earth system processes, such as the biogeochemical cycles of carbon, nitrogen and sulphur (Falkowski et al. 2008, Fuhrman 2009) are driven mainly by marine microbial communities, in which prokaryotes play a fundamental role (Azam et al. 1983, Karl 2002). The current distributions of microorganisms are actually the result of contemporary selection and historical processes. The geographic distance effect should be relatively weak in habitats where dispersal is high, such as in coastal estuaries. However, at the same time, selective factors such as salinity and nutrients are often organized in a gradient in an estuary, tending to produce a distance effect on spatial variation in microbial composition. Tropical estuaries are highly productive and rich in biodiversity and the microbial community in the biodiversity is a central paradigm of the estuarine ecosystems (Venkataraman and Wafar 2005). Microbial communities in the estuaries actively involve and play important roles in a number of nutrient regeneration and biogeochemical cycles. At the same time, these are highly diverse communities which respond rapidly to changing environmental conditions. Hence changes in composition and community structure of nitrifiers can be used as a potential bio-indicator of environmental disturbance (Kowalchuk and Stephen 2001). The adaptability and susceptibility of microorganism may play significant role in nitrification in the nutrient rich and dynamic tropical estuaries (Mosier and Francis 2008). Therefore, knowing the microbial community is a pre-requisite for the systematic study of microbial biogeography and community assembly of nitrifiers in the nitrogen cycle. Spatio-temporal changes in community structure of ammonia oxidizers (AOB and AOA) was studied as these organisms are main players in the rate limiting step of nitrification. Numerous studies based on qPCR analysis of amoA genes have shown AOA to greatly outnumber AOB, in deep oceans and soil (Leininger et al. 2006, He et al. 2007, Mincer et al. 2007, Shen et al. 2008). However, mounting evidence from various estuarine and coastal studies suggests that AOB amoA gene abundance may actually be greater than AOA amoA gene abundance in certain regions of estuaries especially in nutrient rich environments.
For example, Wankel et al. (2011) reported substantially higher AOB amoA gene copy numbers than AOA in Elkhorn Slough estuary, where the AOA amoA gene copy numbers ranged from $4.9 \times 10^3$ to $1.2 \times 10^5$ copies µg$^{-1}$ DNA and AOB amoA gene copy numbers, ranged from $1.2 \times 10^4$ to $4.8 \times 10^6$ copies µg$^{-1}$ DNA. Similar observation on higher abundance of AOB than AOA by two order in nitrogen rich wetlands of China was recorded Wang et al. (2011).

PCR DGGE method was chosen for spatio-temporal studies over other techniques to analyse the diversity of AOB because of the large number of samples despite the possible limitations of this technique (Cilia et al. 1996, Kowalchuk et al. 1997). DGGE gel analysis of AOB showed 4 to 10 predominant phylotypes and AOA showed 7 to 26 phylotypes in the CE. Although there is no marked variation between surface and bottom samples in the band pattern, high number of phylotypes were observed generally in the bottom waters than in the surface waters, which may be due to the re-suspension of ammonia oxidisers from the bottom sediment. The community structure of ammonia oxidizers in the CE could not be compared with other Indian estuaries as it is the first study but it is available from estuarine mangrove ecosystems of India (Krishnan and Bharathi 2009, Das et al. 2013). Hence, the results have been discussed with the available literature from other estuaries in the world. In the clone library of sediment samples of Pearl River estuary, China, 36 OTU were observed for AOA as against 7 OTU for AOB (Jin et al. 2011). Similarly, in agriculturally impacted Elkhorn Slough Estuary, California from 6 to 12 bands was observed for AOB whereas 10 to 24 bands were observed for AOA (Wankel et al. 2011). AOB community in the CE did not show any seasonal variation in the DGGE pattern and 4 to 5 bands were present in the same position in the whole wells of a single gel suggesting the high adaptability of AOB community to varying estuarine conditions. Although microorganisms respond quickly to environmental changes and their community structure are determined by environment, certain level of adaptability towards particular changes is also seen among many microorganisms (Andersson et al. 2006). Community structure stability of AOB population has been reported from Seine estuary, France (Cébron et al. 2004). Unlike AOB, AOA population not only recorded high number of phylotype but also showed temporal variation in diversity in the CE. This temporal
variation of AOA may be due to the less adaptability to varying salinity and nutrient levels in estuary (Liu et al. 2013, Wang et al. 2014). Occurrence of higher DGGE band diversity and richness of AOA compared to AOB have also been reported from Plum Island Sound estuary in USA; Westerschelde estuary in the Netherlands and Bahi’adelTo’bari in Mexico (Beman and Francis 2006, Sahan and Muyzer 2008, Bernhard and Bollmann 2010).

5.5 Phylogeny of AOB

As the Rate recovery study demonstrated that AOB is the major ammonia oxidizers in the water column of the CE, the diversity of AOB was looked in to in the present study. Moreover, phylogeny of AOB is more clearly established than AOA as this organism discovered more than 100 years ago. While studies on the phylogeny of AOA started only after its discovery in 2005, but the classifications and the availability of sequence database are still growing in log phase. Comparative sequence analysis of 40 unique bands of AOB in the CE showed major affiliation of the sequence to uncultured β-proteobacterial AOB. Sequences related to *Nitrosomonas* sp. and *Nitrosospira* sp. were also obtained. Interestingly one band related to γ proteobacteria was also obtained in the sequence comparison. Previous studies have been documented that, among various groups of AOB *Nitrosomonas* sp. and *Nitrosospira* sp. are more predominant in estuarine and coastal environment (Cébron et al. 2004, Cebron and Garnier 2005, Freitag et al. 2005). It has been suggested that freshwater with low oxygen and a high ammonia condition is a possible environmental conditions for dominance AOB with in the *Nitrosomonas* Cluster (De Bie et al. 2001).

5.6 Nitrification Rate

Nitrification is a microbial mediated process that converts ammonium to nitrate via nitrite and occupies a central position within the global nitrogen cycle. Hence, the factors regulating this process are vital to eutrophication as well as to health concerns related to enhanced nitrate levels in aquatic ecosystems (Conley et al. 2009). The observed nitrification rate in the CE ranged from 0.05 to 10.22 µM day\(^{-1}\) and it showed a strong spatio-temporal variation. Nonetheless, the present
nitrification rate is much higher than the previous observation in 2005 in the CE (Miranda et al. 2008). They observed low nitrification rate from a non-detectable level to 3.98 µM day\(^{-1}\) in the CE when the dissolved ammonia concentration was ca 20 µM, but during the present study period (2011) the ammonia concentration reached up 49 µM and the activity increased by three times. This clearly indicates that rise in anthropogenic nitrogen input in the estuary with time. It has been observed that the nitrification rate as well as nutrient input increased substantially in the CE during the past decade. However the nitrification rate (0.05 to 5.4 µM N day\(^{-1}\)) observed in the coastal station (Stn.4) is in close agreement with the recent report of nitrification rate (0.48 to 7.68 µM N day\(^{-1}\)) in the upwelling coastal waters of SW Arabian Sea (Fernandes et al. 2014).

The range of nitrification observed in the CE, was comparable with results obtained from various Indian estuaries like Mahanadi estuary (0.87 µM N \( \cdot \) d \(^{-1}\), when \( \text{NH}_4 = 1.5 \) µM), Narmada estuary (0.82 µM N \( \cdot \) d \(^{-1}\), when \( \text{NH}_4 = 4.0 \) µM) and Tapti (0.42 µM N \( \cdot \) d \(^{-1}\), when \( \text{NH}_4 = 13.0 \) µM) (Sarma and Rao 2013). However nitrification in these estuaries are lower than in the CE at the same time \( \text{NH}_4 \) concentration was also considerably low. Comparable range of nitrification were observed from other world estuaries like Rhone river plume (NW Mediterranean) (up to 2 µM N \( \cdot \) d \(^{-1}\), \( \text{NH}_4 \sim 2.0 \) µM) (Bianchi et al. 1994), Seine estuary in France (up to 16.8 µM N \( \cdot \) d \(^{-1}\), \( \text{NH}_4 \sim 180 \) µM (Brion et al. 2000), Providence River estuary U.S.A (up to 11.6 µM N \( \cdot \) d \(^{-1}\), \( \text{NH}_4 \sim 100 \) (Berounsky and Nixon 1993) and Pearl River Estuary in China (up to 33 µM N \( \cdot \) d \(^{-1}\), \( \text{NH}_4 \sim 350 \) µM) (Dai et al. 2008). However, comparatively higher nitrification rate than the CE, up to 45 µM day\(^{-1}\) has been reported from Schelde estuary in Belgium when the dissolved ammonia concentration up to 150 µM (Bie et al. 2002). More increased in nitrification rate of up to 80 µM day\(^{-1}\) was also recorded in the same estuary in 1984 when the ammonia concentration was 500 µM (Somville 1984). Waste loadings in the Schelde estuary were higher during the first study, which induced higher organic pollution and accompanying oxygen depletion. Rates in the Schelde estuary are still among the highest reported, despite the improved water quality of the estuary. However, in the CE the water quality is deteriorating due to increase anthropogenic inputs. Similar to the abundance of AOB and NOB, the nitrification
rate was also highest during the pre-monsoon and the lowest during monsoon in the CE. A 10 to 40 fold increases in nitrification rate during the pre-monsoon season compared to the monsoon season was observed. The nutrient level in the CE was less during monsoon season due to heavy inflow of rainwater. A previous study on flushing characteristics of the CE showed that the estuary flushes ~42 times a year, and would have freshened many times during monsoon (Revichandran et al. 2012). The increased flushing during the monsoon season along with heavy rain fall may result in dilution of nutrients, (in this case ammonia) and hence the abundance of nitrifiers and their activity would become low during monsoon. Furthermore, the lower residence time of the water in the estuary during peak monsoon play a role in the decreased activity. On the other hand, the discharge becomes inactive during pre-monsoon giving more residence time for nutrients and microorganisms to interact, which results in higher abundance of AOB and NOB and enhanced nitrification rate. Sarma et al. (2012) calculated the relationship between water resident time and nitrification rate from different Indian and world estuaries and found that, the mean flushing time for the Indian estuaries to be <10d, whereas it is < 40d for the estuaries from Europe and USA. Hence, microbes are not able to oxidize ammonium efficiently resulting in low nitrification rates in the Indian estuaries.

5.6.1 Contribution of AOA and AOB towards ammonia oxidation

Many reports are available on the abundance of AOB and AOA in marine and estuarine environments (Crump et al. 1999, Mosier and Francis 2008, Cao et al. 2011) but their relative contribution to ammonia oxidation is hitherto not studied in detail. Both AOB and AOA are present in the CE, the differential contribution of these two groups of organisms to ammonia oxidation is important. Recovered ammonia oxidation rate assay in the presence of specific antibiotics was used to understand the contribution of AOB and AOA in nitrification. Ammonia oxidation activity of about 50–75% could be recovered in the water sample after removing acetylene gas, which confirms the active recovery of ammonia oxidation. It was observed that 40–65% of ammonia oxidization activity was contributed by AOB from the water samples supplemented with archaeal protein inhibitors. On the
other hand, the contribution of AOA was considerably low in the CE as the recovered ammonia oxidation rate was reduced to 15–45 % in the water treated with bacterial protein synthesis inhibitors. No significant difference was noticed in the relative contribution of AOA between surface and bottom waters, whereas spatial differences were observed. Maximum recovery of AOA mediated ammonia oxidation was observed in the surface and bottom waters at station 2 (45 %) while it was minimum in the bottom water at station 3 (14%). AOB-mediated recovery of ammonia oxidation rate was < 50 % in the surface and bottom waters at station 1 and 2, while it was >50 % at station 3. Although both AOB and AOA harbour ammonia monooxygenase gene, the structure and mode of action of the respective enzymes are different. Archaeal ammonia monooxygenase gets triggered at lower concentrations of ammonia and switches off at higher concentrations, while that of bacterial gets triggered at higher concentration of ammonia (Bernhard et al. 2010). In the CE, concentrations of ammonia were high close to 50–65 % of the dissolved inorganic nitrogen which is conducive for AOB. However it may vary depending on the system. Our results are in agreement with the recent study in Colne Estuary, United Kingdom (Li et al. 2015). Similar observation has been reported from terrestrial ecosystems. Taylor et al. (2010) reported variation in the dominance of either bacterial or archaearcha or both in different soil system. Nitrification driven by bacteria and less contribution of Archaea was reported by (Di et al. 2009) in nitrogen-rich grassland soils. Similar results were also reported in Zinc contaminated soil system by (Mertens et al. 2009).

### 5.6.2 Inter parameter relationships

As for ‘the environment selects’ have shown a significant correlation between microbial composition and at least one measured environmental variable (availability of resources such as nutrients and dissolved organic carbon) or habitat feature F (physical parameters such as temperature and salinity) (Kamke et al. 2010, Agogué et al. 2011, Campbell et al. 2011). Nitrification rate in general, is categorizes under ‘the environment selects’ as it is regulated by many factors including salinity (Santoro and Enrich-Prast 2009), \( \text{NH}_4 \) (Triska et al. 1990, Jones Jr et al. 1995), pH (Sarathchandra 1978), temperature (Jones and Hood 1980), oxygen.
concentration (Stenstrom and Poduska 1980, Triska et al. 1990), competition for NH$_4$ (Verhagen and Laanbroek 1991), and organic carbon availability (Verhagen and Laanbroek 1991). Nitrification also depends on NH$_4$ regeneration rates, which in turn is positively influenced by temperature (Nixon 1981). In the present study, simple correlation analysis and PCA analysis were employed to elucidate the factors governing the nitrification rate. The results of the regression analysis are given in Table 4.10. Seawater influx, i.e salinity, is considered as one of the major factors controlling nitrification process in many estuaries (Stehr et al. 1995, Rysgaard et al. 1999, Mosier and Francis 2008). However, differences in the optimum salinity for nitrification rate has been reported from many estuaries; for example low salinity (0 to 5) in Barataria Bay estuary in Mexico (Jones and Hood 1980) and high salinity (25 to 35) in Douro River estuary in Portugal (Magalhães et al. 2005). In the CE, although no significant correlation between salinity and nitrification rate was seen, high activity was observed at the intermediate salinity waters. The similar result has been reported in the CE previously by Miranda et al. (2008). This is also in agreement with reports from other estuaries like Scheldt estuary in Netherlands (Andersson et al. 2006) and Fjord estuary in Denmark (Rysgaard et al. 1999), where high nitrification was observed at intermediate salinity of 10 to 20. Temperature has been shown to be a major factor controlling the seasonal variations in pelagic nitrification (Berounsky and Nixon 1993). For example, the nitrification rate in Narragansett Bay ranges from near zero during winter to $\sim$1µM N L$^{-1}$ d$^{-1}$ during summer, with an apparent Q10 $\sim$6.8 (Q10 represents the increase in the rate of a process at each 10°C increase in temperature (Berounsky and Nixon 1990). However in the present study no clear response of nitrification to the relatively small variation in temperature was detected.

Nitrification rate in the CE was largely controlled by ammonia levels ($r = 0.65, p < 0.01, n = 28$), which in turn is regulated through freshwater discharge (anthropogenic inputs) and flushing. This is in agreement with previous reports from Elbe estuary in Germany (Stehr et al. 1995), Seine estuary (Cébron et al. 2003) and Urdaibai estuary (Iriarte et al. 1997). The spatio-temporal variation in nitrification rate ($r<0.8, p<0.01, n = 24$ except $N. mobilis$), was also limited by AOB and NOB abundance. Majority of the earlier studies on nitrification rate did not
consider the role of the nitrifying organisms in the process (Somville 1984, Berounsky and Nixon 1993, Feliatra and Bianchi 1993, Bianchi et al. 1994, Brion et al. 2000), and the limited study on the nitrifiers abundance was based on culture dependant (Dai et al. 2008). Recently, a couple of ecological studies have dealt with nitrifiers abundance and phylogeny. However, interestingly majority of these studies did not studied nitrification rate or if studied it was only on the potential nitrification rate (Cao et al. 2011, Smith et al. 2015). This potential nitrification rate is not comparable with in situ nitrification as the rate estimation is carried out non-limiting substrate and oxygen concentration. Therefore the relationship between nitrifiers abundance and nitrification rate in the present study is compared with few estuaries and coastal waters. Significant positive correlation observed between nitrification rate and nitrifiers abundance in the CE was in agreement with these estuaries and coastal system (Beman and Francis 2006, Caffrey et al. 2007, Beman et al. 2008, Smith et al. 2014). The correlation between nitrification rate and DIN, nitrification rate and nitrifiers abundance and between DIN and nitrifiers abundance suggest that the dominant process affecting DIN dynamics in the CE is nitrification. Intense nitrification in estuaries of large rivers receiving important ammonia inputs is a general observation (Brion et al. 2000). High turbidity in the CE may also enhance the intense nitrification rate. This is mainly due to the close association between nitrifying organisms and particles (Helder and De Vries 1983, Owens 1986) and thereby providing optimal substrate concentrations and habitat for estuarine nitrifiers (Balls et al. 1996). Moreover, turbidity can also reduce the inhibitory effect of light (Merbt et al. 2012). In CE, significant statistical correlation between SPM and nitrification was not observed as SPM was high throughout and did not show any variability. Similarly, O₂ did not show any relationship to nitrification rate in the CE as the water column is well oxygenated and its much above the oxygen requirements of nitrifiers (Garnier et al. 2007).

The present study reports for the first time the spatial and temporal variation in the abundance and activity of nitrifiers from the CE, a monsoon driven nutrient rich tropical estuary along the southwest coast of India. The variability in temporal and seasonal patterns indicates a complex relationship between physico-chemical and biological controlling factors. It was observed that the levels of
ammonia in the water column have significant influence on the abundance of AOB, NOB and nitrification rate. Recovered ammonia oxidation rate experiment suggests that, though both AOB and AOA contributed in ammonia oxidation in the CE, AOB is the major player in nitrification. As AOB are more adapted to varying environmental conditions of the CE compared to AOA. From the study it could be concluded that in the CE, a monsoon driven estuary, the nitrification rate and microorganisms involved are greatly influenced by seasonal variation brought in by river water discharge and flushing. Though nitrification rate was found to be increasing with increased nutrient concentration in the CE, the anthropogenic inputs have to be controlled to prevent eutrophication and associated environmental changes.