5.1 Metal Pollution

Heavy metal pollution is an ever increasing problem in oceans, coastal waters, estuaries, rivers and lakes. Estuaries, which form an important component of the coastal ecosystem, are also known to be the major reservoirs of trace metals, both from anthropogenic and natural origins (Bryan, 1980; Langston, 1982). The concentration of heavy metals in CE was higher than that of the other impacted Indian estuaries (Qasim, 2003; Zingade and Desai, 1981; Subramanian et al., 1988; Mukherjee and Kumar 2012; Kumar and Edward 2008; Banarjee and Gupta, 2012) except few regions close to Mumbai. Heavy metals reaching the estuaries through fresh water/effluents may get trapped in the sediment and their transportation towards the marine environment may take a long time depending on the speed and course of water currents. Estuarine sediments represent one of the ultimate sinks for heavy metals discharged into the environment (Bettinetti et al., 2003; Hollert et al., 2006). During the past three decades in CE, Zn concentration in the sediments have increased from 70 to 1266 mg kg⁻¹ (Venugopal et al., 1982; Balachandran, 2006). Similarly, the Cd concentration also showed an increase from 1.7 to 14.94 mg kg⁻¹ during 1990-2000 (Nair et al., 1990; Rajamani Amma, 1994; Balachandran et al., 2006). The significant increase in the heavy metal concentration recorded over a period of time in CE (Balachandran et al., 2005; Nair et al., 2006; Paul SK, 2001) is mostly through increased discharge of industrial and domestic wastes into the estuary (Balachandran et al., 2006; Nair et al., 2006). Indiscriminate release of industrial effluents and domestic wastewater resulted in similar increase of metals in Ulhas estuary and the nearby Thane Creek (Patel et al., 1985; Zingde and Desai 1981; Sahu and Bhosale, 1991; Ram et al., 1998). An increase in
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metal concentration from bottom to the surface of the sediment core in the CE was observed (Unnikrishnan and Nair, 2004). This rise is mainly due the increased anthropogenic activities with time. The increased monsoonal supply of anthropogenic inputs together with previously deposited sediments may be the reason for the increased metal contamination in the CE. Further, residual effects of most of these heavy metals on aquatic biota are long lasting and highly deleterious (Forstner et al. 1979; De Flora et al., 1994) as they are not easily or rapidly eliminated from these ecosystems by natural degradation processes. In CE, the concentration of different metals varied in sediment and water. Metal concentration in CE was in the order of Zn > Ni > Cd > Cu > Co at all the stations. The concentration level of different metals is influenced by the type of effluents discharged by industries. Hg concentration was below the detectable limit in the CE though considerable enrichment of Hg was evident in the estuary of Mumbai compare to the open shore coastal water off Bassein–Mumbai (Zingde and Desai, 1981; Sahu and Bhosale, 1991). The anthropogenic input of Hg in the estuary is mainly from chloro-alkali production, instrument manufacturing and dentistry which are absent in the industrial belt of the CE. Among the metals studied, Zn concentration during this study was very high such high concentration of Zn has been reported earlier in the CE (Venugopal, 1982; Balachandran, 2006). Spatial variation of metal concentration in surface sediments of the estuaries is often attributed to mixing of sediments from different origins and to pollution sources (Forstner, 1981). Station-wise variation in metal concentration was observed in the present study. Eloor recorded the highest pollution compared to Vypin. The level of metal concentration varies depending on the distance from the point of discharges. Being mostly industrial in origin, this high concentration of certain metals at Eloor can be attributed to its closeness with the industrial belt. The concentration of metals in the CE was high in the sediment compared to water at all the stations. The higher levels of the metals in the sediment is due to the accumulation of metals through the complex physical and chemical adsorption mechanisms, which depend on the nature of the sediment matrix and the properties of the adsorbed compounds. The high affinity of the metals with organic matter, metal oxides, and clay minerals helps in accumulating them in benthic sediment effectively over time (Maher and Aislabie, 1992; Ankley et al., 1992; Wang et al.,
The concentration of the metals in the water column seems to depend on the metal concentration in the sediment. It was also observed that the concentration in the CE water was influenced by the level of the respective metal concentration in the sediment. The favourable physico-chemical conditions of the sediment could have remobilized and released the metals into the water column in the CE. The present results also confirm that the CE is facing serious metal pollution problem which is increasing with time due to enhanced discharge of untreated/partly treated industrial effluents.

5.2 Effect of heavy metal pollution on bacterial abundance

Heavy metals are both naturally and artificially present in ecosystems and many metals and metalloids (e.g., Zn, Cu, Mn) are essential in the functioning of living organisms as micronutrients serving as structural proteins and pigment, used in the redox processes, regulation of the osmotic pressure, maintaining the ionic balance and enzyme component of the cells (Kosolapov et al., 2004). The high-surface area-to-volume ratio provides a large contact area for the microbes to interact with metals in the surrounding environment (Ledin, 2000). Since microbial encounters with metals are unavoidable in the environment, it is not surprising that microbes have developed suitable means to put to use some metals that can exist in more than one oxidation state, for their benefit as electron donors or acceptors in their energy metabolism (Ehrlich, 1997). A feature of heavy metal physiology is that even though many of them are essential for growth, they are also reported to have comprehensively toxic effects on cells, mainly as a result of their ability to denature protein molecules (Gadd and Griffiths, 1977). Thus, metals have a high ecological significance due to their toxicity and accumulative behavior. The xenobiotic pumps in microbes are also involved in heavy-metal detoxification (Bard and White, 2000) that poses a serious threat to the environment. The distribution of metals in the environment are at times depends on the microbes. Effect of heavy metal pollution on the abundance, structure and diversity, and dynamics of microorganisms in the marine environment is a topic of growing environmental concern as it has direct and long lasting impact on the ecosystem functioning and are not easily degradable (Valsecchi et al., 1995; Bong, et al., 2010).
In marine environment, microorganisms are exposed to metals ever since their origin and have acquired several mechanisms to survive in such environments. Within the aquatic system, heterotrophic bacteria are the most abundant and ubiquitous forms, and is recognized to be a critical component of the marine biogeochemical cycles and food web dynamics. There are several studies in which it has been reported that heavy metal pollution can affect the abundance and diversity of microbial population (Ellis et al., 2003; Kandeler et al., 2000; Khan and Scullion, 2000; Chen et al., 2013; Wang et al., 2005; Wang et al., 2007). The total and viable counts in sediment and water of the CE were within the reported values from other Indian estuaries despite being considered as metal polluted estuary. Earlier study by Thothalil (2008a) had reported the abundance of bacteria in the CE to be $10^9$ L$^{-1}$ which was the same reported for the Madovi and Zuari estuaries (Ram et al., 2003). In CE there was no significant difference in bacterial abundance between stations like Eloor with high metal concentration and Vypin with low metal concentration, except for the TCC which was low in number at the polluted station of Eloor. Eloor recorded the highest concentration of 1159 mg L$^{-1}$ of Zn where the respective abundance of TC, TVC and TCC in water were $10^9$, $10^5$ and $10^4$ cells L$^{-1}$, clearly showing that the abundance of bacteria was as commonly observed in any tropical estuary and was not significantly affected by the metal concentration. Bong et al., (2010) also observed that higher concentration of Zn did not cause any significant change on the bacterial abundance. An unchanged bacterial abundance was also observed by Liu et al., (2012), who reported no differences in bacterial abundance which was subjected to long-term heavy metal pollution. Chen et al (2013) suggest that long-term heavy metal pollution had no effect and did not decrease the microbial biomass, activity and diversity. Another explanation for maintaining the uniform abundance at the three stations may be that metal contamination would have brought about a shift within the soil microbes from sensitive to less sensitive microorganisms (Maliszewska et al., 1985; Giller et al., 1998). Some group may be eliminated whilst others showed increase in abundance because of reduced competition for substrate (van Beelen and Doelman, 1997). The replacement of sensitive bacteria by the resistant ones may not result in any net effect on broad microbial indices such as soil respiration or total
biomass. Based on this study, it can be concluded that in the CE metal concentration in sediment and water, whether high or low, had no significant influence on total and viable bacterial abundance.

5.3 Studies on Metal resistant Bacteria (MRB)

5.3.1 Abundance

Population studies on metal resistant bacteria are very limited and are mostly enumerated in a given metal amended medium but presence of MRB have been shown to be ubiquitous as they have been isolated from different geographical locations (Mudryk 2005). Azam et al., (1983) have reported that an adaptation or selection of bacteria to heavy metal naturally occurs in the marine environment. And marine bacterial strains can tolerate (without pre-adaptation) five- to tenfold higher heavy metals concentrations than in seawater (Sigel 1993) as bacteria not only have a high affinity towards metals but can also accumulate both heavy and toxic metals by a variety of mechanisms (Harrison et al., 2006; Jeyasingh and Philip, 2005). The abundance of metal resistant bacteria is more in marine system since the marine isolates are encapsulated strains. It seems quite reasonable to state that many marine bacteria having high metal tolerance produce metal sorption polysaccharides (Sigel 1993). The occurrence of MRB in the CE is not surprising and it was an order less than TCC. TCC was always higher than MRB and decreased with increase in the concentration of metal whereas MRB increased with metal concentration. This may be due to adaptation as mentioned earlier by horizontal transfer of resistance genes located on conjugal plasmid (Rasmussen and Sorensen, 1998). It appears that MRB population in CE may be long term adaption of the TCC. In the CE, regardless of the number of the metals present, the MRB abundance was in the order of $10^2 - 10^4$ g$^{-1}$ with sediment recording higher population. MRB population was one order less than TCC. Spatial variation in the abundance of MRB in water and sediment was observed in the CE which correlated to the concentration of the metal. In polluted Eloor sediment, MRB was high and ranged between $2.51 \pm 0.13 \times 10^4$ to $6.3 \pm 0.4 \times 10^2$ cfug$^{-1}$ compared to other two stations. Comparable population of MRB have been recorded along the Indian coast and higher population was in sediment than in water (Ramaiah and De
They also showed that the abundance varied between regions and was dependant on the concentration of the metal with Mumbai recording higher population. Higher abundance in water was also recorded by Ivanova et al., (2002). They found the percentage of the metal-resistant strains isolated from seawater (50% of the strains studied) was higher than mollusks and seaweeds (26 and 11%, respectively) and varies with the source. In CE, the population of MRB was high in sediment as the concentration of the metal in the sediment was more than double in the water. This high concentration may be the reason for the evolution of more resistant bacteria in sediment. In Torch Lake copper resistant fraction of the surface community represented only 1/100th of the cultivable community, but below the surface sediment, the viable cell count dropped and copper resistant bacteria increased to 30–75% of the cultivable cells (Konstantinidis 2003). Earlier study on Hg resistant bacteria from the coastal waters of India has shown that MRB abundance vary from 98% to as low as >0.5 % (Ramaiah and De 2003). They reported the occurrence of Hg MRB from no-pollution (Positira, Marmugao, Terekhol, Gopalpur), low-pollution (Malvan, Karwar, Paradip, Nagapattinam), and high-pollution (Mumbai, Chennai, Mangalore, Kulai, Padubidri, and Ratnagiri) coastal locations showing that retrieval depends on the location and the metal concentration used for isolation (Ramaiah and De, 2003). In CE, Hg resistant population was also recorded though the metal was below detectable levels at Eloor. The population of Hg resistant bacteria was $10^2$ L$^{-1}$ in water and $10^3$ g$^{-1}$ in sediment. Studies using microcosm have shown that the immediate response to metal pollution leads to a rapid increase in the frequency of resistant bacteria but on a long term there is adaptation to heavy metal exposure and maintenance of the hemostats. Microbial communities have been used to reveal the long-term consequences of heavy metal contamination, report positive correlations between environmental and metal concentration and increased tolerance of microbial community (Lock and Janssen, 2005; Diaz-Ravina et al. 1994; Pennanen et al. 1996). The common finding is that bacteria have a high frequency of resistance to any stress (Andersson 2003; Martinez et al 2009; Stortz et al., 1990; Bjedov et al 2003). These MRB bacteria, would have developed resistance to metals by different mechanisms, such as metal sorption and intra-/ extra-cellular sequestration (Gadd, 2004 Hamamura)
et al., 2009; Wuertz et al, 1997), detoxification and efflux pump systems to protect themselves from metal toxicities (Silver and Phung, 1996 and 2005; Teitzel et al., 2006; Nakagawa and Takai 2008; Haferburg et al., 2009; Martinez et al., 2009; Haferburg and Kothe, 2010) to survive in stress environment. Microbial communities can be used to reveal the long-term consequences of heavy metal contamination, reporting positive correlations between environmental metal concentration and increased microbial community tolerance and it can also give the sources of their isolation (Lock and Janssen, 2005; Diaz-Ravina et al. 1994; Pennanen et al. 1996). Since MRB do not arise by chance and that there must be selection factors (Hideomi et al., 1997) and in CE, the selection factor is the heavy metals. The presence of MRB is important in the contaminated CE for continuing the basic biological processes (De et al., 2003)

5.3.2 Multiple resistances

Most of the studies on metal resistant bacteria are based on the study on individual bacterium, its MIC and mode of resistance (Hassen et al 1998; Rasmussen and Sorensen1998; Konstantinidis et al., 2003). Bacteria tolerate low concentrations of certain transition metals such as cobalt, copper, nickel and zinc as they are essential for many cellular processes of bacteria. Higher concentration may be cytotoxic and all the bacteria may not tolerate metals. Most of the isolates from the CE were tolerant to multiple metal ions. Such multi-tolerance to metals is a common phenomenon for both algae and bacteria in aquatic system (Takamura et al., 1989). Multiple tolerance to more than 5 metals has been observed in halophilic eubacteria from solar salterns of Spain (Nieto et al.,1989). The percentage of resistance varied with metal and location. It was observed that 90 – 100% of the bacteria retrieved from Eloor region were resistant against 5 mM concentration of Zn, Co, Ni and Cu, 50 – 60% was resistant against Cd and 20-30% was resistant against Hg. At Vypin and Munambam metal tolerance was less than 40%. Studies on the cultures of Oregon soils showed tolerance to heavy metals with a large proportion tolerating Ni, Pb, and Zn upto 20 mM. The patterns of tolerance among cultures varied as in the case of those in the CE. The resistance depended on the metal concentration. The high levels of resistance and the widespread tolerance were attributed to the high metal contents (4,390 mg Ni kg⁻¹ and
Studies on toxicity of metals to bacteria frequently report that the bacteria have a wide range of resistance mechanisms to—and intracellular uptake of—trace metals from seawater (Ross, 1988; Ferris et al., 1999). For example, cadmium tolerant communities are likely to show co-tolerant to Zn (Paulsson et al., 2000) and organo tin tolerance simultaneously occurs associated with cadmium tolerance (Suzuki et al., 1992). Ivanova et al., (2002)suggest that the tolerance of bacteria to a high concentration of a particular metal does not always correlate with their tolerance to other metals. This may be due to the existence of different mechanisms responsible for bacterial tolerance to heavy metals (Nies,1999,2000, 2003 2007). These mechanisms may be encoded by chromosomal genes, but more usually loci conferring resistance are located on plasmids (Cervantes and Gutierrez-Corona,1994; Wuertz et al 1997). A few metals such as lead, cadmium, mercury, silver and chromium with no known beneficial effects to bacterial cells are toxic even at low concentrations (Nies, 2003). Mercury resistance was not observed in bacterial isolates from both Vypin and Munambam. It is interesting to note that 20 - 30% of the microbes retrieved from both soil and water samples of Eloor exhibited resistance against Hg, though it was not detected in any of the samples. This may be due the ability of the same microorganism to be resistant to one or a group of heavy metals (Allen et al. 1977; Barkay et al. 1987; De et al., 2003; Silver and Phung, 1996; Timoney et al. 1978) or the natural flora is adapted to Hg resistance (Ramaiah and De , 2003). In the CE, almost all the isolates from water and sediment samples of Eloor were resistant upto 0.5 mM AgNO₃.A significant number of Eloor isolates from water (~30 %) and sediment (~40 %) showed resistance upto 250 and 1000 mM, respectively. However, Vypin and Munambam isolates showed resistance to AgNO₃ only up to a concentration of 1 mM. Bacteria from Vypin and Munambam were the least resistant to AgNO₃ and only less than 40 % of isolates showed any resistance up to 0.5 mM. This low resistant compared with other metals, may be due to silver higher toxicity to microorganisms (Zhao and Stevens 1998) as silver and its compounds have strong inhibitory and bactericidal effects. (Franke et al. 2001;;Lok et al. 2006; Cho et al. 2005; Silver 2003). The resistant to silver may be due nontoxic NPs. It has been shown that thesilver resistant \textit{Pseudomonas stutzeri}
AG259 accumulates silver particles of particle size 35 to 46 nm, in their cell (Slawson et al., 1992). However, the mechanism by which these microorganisms as such cell or their products reduce metal ions to non toxic NPs is hitherto unknown. The possibility of biological precipitation of the metal compounds in the periplasmic space is suggested (Xie et al., 2007). The possibility of efflux pump in tendering resistant cannot be ruled out. As the efflux pumps of gram negative bacteria are not specified for a particular compound, the same pump can function for extruding excess concentrations of any heavy metals or antibiotics or other compounds which are toxic to bacteria (Silver and Phung, 1996; Ramos et al., 2002; Pumbwe et al., 2007; Martinez, et al., 2009). The number of isolates resistant to multiple metals was high in the sediment compared to water in the CE which may be due to exposure to different heavy metals that are available either in solution or adsorbed on soil colloids (Gilleret al., 1998) as discussed earlier. The concentration of metals in CE increased from Vypin to Eloor in sediment and water which corroborated with high, percentage of multiple metal resistant bacteria at the gross polluted site compared to the intermediate and less polluted stations. It is evident from this study that the heavy metal pollution in CE propagated the evolution of multiple resistant bacteria and the percentage of MRB resistance in sediment could serve as a reliable indicator of environmental status and level of pollution than water (Caccia et al., 2003). Krishnan et al., (2007) reported that both autochthonous autotrophs and heterotrophs work in tandem in reducing Mn and other related metal ions in mangrove sediments.

5.3.3 Co-occurrence of antibiotic resistance

Metal contamination functions as a selective agent in the proliferation of antibiotic resistance (Baker-Austin et al., 2006). MRB of the CE also demonstrated resistance towards the antibiotics which belonged to different chemical classes. Interestingly, 93.9 % of metal resistant microbial isolates had resistance to at least one antibiotics used. In the CE, the antibiotic resistance was more prevalent among metal resistant bacteria isolated from metal rich Eloor station and more in the sediment than in water. More than 50% of the isolates from Eloor showed resistance against antibiotics such as A, At, Ak, C, Cf, Na, Nf, T and Va. Fifty percentage of MRB isolates from Eloor showed MAR index of 0.25 with water showing higher MAR index against antibiotics A,
At, Ak, C, Cf, Na, Nf, T and Va compared to the less polluted isolates (10 %) from Vipin. The percentage of isolates with higher MAR index decreased with sediment recording higher index than water. Eloor showed higher MAR index compared to the other 2 stations a trend seen with multiple metal resistances. Such strong patterns of co-occurrence between metal and antibiotic resistance in environmental settings have been reported (Baker-Austin et al., 2006; De Souza et al., 2006; McArthur et al, 2011), including soils amended with Cu (Berg et al., 2005), freshwater microcosms amended with Cd and Ni (Stepanauskas et al., 2006), and liquid pure cultures containing Cu and Zn (Caille et al., 2007). There is substantial overlap between known mechanisms for metals and antibiotic resistance, such as those for copper and tetracyclines, copper and ciprofloxacin, and arsenic and b-lactams (Baker-Austin et al., 2006). Exposures of microbes to metal have been shown to increase the incidence of bacterial antibiotic co-resistance through the transfer of genetic elements containing both metal and antibiotic resistance genes and also through the selection of organisms that contain elements, such as non-specific efflux pumps, which can convey cross-resistance to both metals and antibiotics (Summers 2002). Jacoby (1974) studied properties of R-plasmids determining gentamicin resistance by acetylation in *P. aeruginosa*. He found that plasmids also determine a number of other properties not previously known to be associated with *Pseudomonas* R-factors, such as resistance to ultraviolet (UV) light, to Hg2+, and to organic mercurial. It has been observed that bacteria which carrying resistant to metals are also resistant to many antibiotics and other toxic chemicals by virtue of carrying plasmids and/or transposons encoding genetically linked metal and antibiotic resistances. The gene cascade responsible for metal resistance and antibiotic resistance reside in the same mobile genetic platforms, resulting in the co-expression of resistance to multiple antibiotics and metals (Novick and Roth, 1968; Foster, 1981; Baker-Austin, et al., 2006). Study by Ramaiah and De (2003) on MRB from Indian coastal waters indicated that increased use for industrial and agricultural practices and the subsequent effluent discharges into marine regimes continuously increased metal concentrations. This might lead to the selection of microbial assemblages capable of high tolerance to metal through acquisition of plasmids and/or transposable elements. Co-resistance is a potential mechanism of dual resistance in bacteria based culture result. Co-resistance was found to
multiple metals and multiple antibiotics which suggests that co-selection is not limited to a subset of metals or antibiotics in the CE. The co-resistance may be attributed to the genes located together on the same genetic element such as a plasmid, transposon or integron (Novick and Roth, 1968, Baker-Austinet al., 2006, Foster,1981; Chapman, 2003). The microhabitat sediment served as sources of metal and antibiotic resistance. It is plausible that this elevated tolerance is a result of higher selective pressure imposed upon microbial communities by elevated metal concentrations in sediments compare to water within the CE.

5.3.4 Enzyme expression profile

Marine organic compounds provide a labile energy and carbon source to heterotrophic bacteria. The bacterial degradation of high molecular-weight organic compounds is initiated by the activity of extracellular enzymes (Hoppe and Gocke 1993; Chrost 1991). Enzymes are largely responsible for marine biogeochemical cycling, due to the important and essential functions in various biochemical processes in marine ecosystems. Breakdown by bacterial enzymatic hydrolysis is a crucial first step in bacterial production to yield sufficiently small monomers from macromolecules of polymeric organic matter (Hoppe1991) enhancing regeneration of dissolved organic matter, nutrients, and trace metals from particulate matter and organic aggregates (Azam et al 1983). Enzyme activities, however, are quite sensitive to physicochemical and environmental parameters, including metals (Revilla et al., 2005). Since heavy metals severely affect the growth, morphology and metabolism of microorganisms through functional disturbance, protein denaturation and destruction of the integrity of cell membranes (Leita et al., 1995), heavy metals would directly affect the diversity, activity and the community structure of the microbial population (Ellis et al., 2003; Kandeler et al., 2000; Khan and Scullion, 2002). At low contamination level, activities of some enzymes were least affected but significantly decreased the activities of some other enzymes (Kandeler et al., 2000) which however, depended on the metal. For instance, at high concentrations of Zn, the aminopeptidase activity is inhibited, but no change is seen for trypsin and chymotrypsin (Bong et al., 2010). In the CE, the concentration of Zn at Eloor was as high as 2758 mgkg⁻¹ in the sediment and 1159 mgL⁻¹ the water and is apparent that this high concentration would be having an overall effect
on the activities of enzymes. It was found that Zn inhibited aminopeptidase an important enzyme in the nitrogen cycle (Choudhury and Shrivastava, 2001). Though aminopeptidase was studied in CE, Zn contamination would have also affected the biogeochemical cycle in the CE. For the stability and co-existence of species in different environmental conditions, bacteria trade-off abilities to perform one set of function for another (Bohannan et al., 2002; Tilman, 2000). Therefore, it can be reasonably concluded that the communities inhabiting the heavily polluted locations are constrained in the functional diversity. Recent studies by Jessup and Bohannan (2008) have shown that environmental changes can alter trade off shape, and the different physiological mechanisms can lead to different sensitivities to environmental changes. In relatively pristine Antarctic waters, De Souza et al.,(2006) have reported trade off mechanism among bacteria with respect to multiple enzymes and multiple metal resistances. It was observed that 75 – 100% of the organisms retrieved from water and sediment samples of Eloor region had low expression profile of amylase, protease, lipase and gelatinase enzymes. The inhibition of proteolytic enzyme observed in this study is consistent with the previous report on toxic effect of Zn at high concentration that could involve masking of the catalytically active subunits of the enzyme or substrate proteins, changing the conformation of the enzyme structure and competing with cation activators connected with the formation of a substrate enzyme complex (Silver and Ji, 1994). It should be noted that ~40% of the organisms isolated from soil samples of Vypin region had high protease enzyme expression profile and 25 – 35% of isolates had high expression profile of amylase, lipase and gelatinase enzymes. In the CE, the heavy metal pollution significantly influenced the enzyme expression profile of the same species of microorganism isolated from different sampling sites. It is known that enzymatic hydrolysis of large dissolved and particular organic matter to micro-molecules of less than 600 Da is the vital process in sustaining primary productivity in the marine environment (Hoppe et al., 2002; Yamada and Suzumura, 2010). Therefore, it can be assumed that the reduced enzyme expression profile of microorganisms in Eloor region may be adversely influencing the biogeochemical cycle and productivity in that region. However, further studies by integrating the molecular and biochemical tools
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are required to explain the correlation between heavy metal induced repression of microbial enzyme profile and productivity in the CE.

**5.3.5 Diversity of MRB**

A primary objective within microbial ecology is the structuring of communities on local and global scales (Jessup et al., 2004). In recent years, culture-independent methods have been used in preference to traditional isolation techniques for microbial community analysis. However, there is reservation regarding if uncultured organisms are important for understanding the impact of metal on indigenous bacteria. This study was based on bacteria isolated by traditional plate culture methods, as readily culturable bacteria may be the most important in terms of both biomass and activity. Ellis et al., (2003) found that metal contamination did not have a significant effect on the total genetic diversity present but affected physiological status, so that the number of bacteria capable of responding to laboratory culture and their taxonomic distribution were altered. Thus, it appears that plate counts may be a more appropriate method for determining the effect of heavy metals on soil bacteria than culture-independent approaches.

In the CE, Gram negative and positive MRB were present. Sequestration of heavy metals in cell wall is a common strategy in gram negative and positive organisms (Haferburg and Kothe, 2007; Haferburg, et al., 2009). Because these metal resistance determinants are commonly located on plasmids or on transposons, it has been suggested that these genes may be spread to divergent bacteria by horizontal transfer and there are evidences to show that these genes are shared both within as well as across the gram positive and gram-negative bacteria communities (Bogdanova et al., 1988). The major MRB groups in the CE were Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Proteobacteria and Firmicutes were present at all the stations. However, the number of groups in the CE was less than that in the Palk Bay (southeast coast of India). Nithya et al., (2011) recorded Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria (including alpha (α)-, gamma(γ)-, delta(δ)-, and epsilon(ε)-Proteobacteria), and uncultured bacteria in the Palk Bay sediments. The decreased in diversity may be due to the high level of metal pollution in the CE. Heavy metals are both naturally and artificially present in the aquatic ecosystems and
have a high ecological significance due to their toxicity and accumulative nature. Contamination of soil with heavy metals affects the qualitative and quantitative structure of microbial communities resulting in decreased metabolic activity and diversity (Giller et al. 1998; Del et al. 1999).

There was spatial variation in the diversity of MRB, the Gram negative MRB being more prevalent (64.2%) and increase of Gram negative bacteria with the level of metal pollution. In Eloor the percentage of Gram negative bacteria was 86.7% whereas in Vypin it was 47.5%. Studies have shown that gram-negative bacteria are the dominant species in metal contaminated soils (Doelman and Haanstra, 1984; 1986; Mata et al., 2012) as well as in regions of heavy metal occurrence such as deep sea sediment and vents (Campbellet al., 2001; Haddad et al 1995). Highly specific efflux pumps assist Gram negative bacteria to regulate their intracellular metal concentration even if they are not specialized to grow in the presence of high concentrations of heavy metals (Martinez, et al., 2009). The presence or absence of a metal ion transporter of particular specificity depends on the metal ion, bacterial species and physiological status of the cell (Nies and Silver 1995). Concentration of heavy metals significantly influenced the diversity of cultivable bacteria in the CE. At Eloor, the overall diversity in water and sediment at group level was low compared to that at Vypin and Munambam. Bacteriodetes and Actinobacteria were observed in less polluted Munambam and Vypin. The diversity decreased at generic and species levels with increased metal concentration in the CE. At Eloor, the number of genus and species was less compared to that at Vypin. High Shannon indices (H’) of 3.1 and 3.3 in water and sediment respectively were recorded at Vypin. A contrasting perspective has been recorded from sites contaminated with multiple heavy metals. Gillan et al., (2005), investigating the chronically polluted sediments within Norwegian fjords it has been observed that elevated concentrations of Cd, Cu, Pb and Zn did not significantly affect microbial community diversity along a concentration gradient. Similarly, Sorci et al. (1999) employed 16S rRNA to monitor the bacterial diversity within metal and organically polluted sites of New Bedford Harbor, Massachusetts and recorded elevated microbial diversity within the contaminated sites, relative to reference sediments. Muller et al., (2001) have reported that most of the dominant
genera that occurred at the less polluted site were not observed at the grossly polluted site because metal pollution can cause shifts in the composition of the bacterial community. Metals had a significant impact on microbial community structure (Wang et al., 2007). Sites contaminated by a single metal species such as mercury (Rasmussen and Sorensen, 1998) or cadmium (Ganguly and Jana, 2002), showed low microbial diversity. This is due to the distinctive selective pressures of the metal, encouraging the growth of specialist metal-resistant organisms under a certain group. Proteobacteria was dominant in the grossly polluted station (Eloor). Genome analysis of marine Proteobacteria has revealed that these organisms have an array of metal transport systems including detoxification mechanisms, depending on the bacterial strain and the source of isolation, which facilitates their survival in an environment containing elevated concentrations of heavy metals (Nakagawa et al., 2007). Diversity of Proteobacteria retrieved from Eloor included 18 spp, whereas only seven were recorded at Vypin. Previous studies have noted the presence of Gamma Proteobacteria in sediments contaminated with metal concentrations (Feris et al. 1999). The high tolerance of marine Proteobacteria to heavy metals has been reported earlier and the mechanism has been attributed to the high concentration of polysaccharides present in them (Ivanova et al., 2001). Another study showed that thiolate peptides may be the primary regulators of cadmium homeostasis in marine Proteobacteria (Ivanova 2002). Further studies have reported detoxification of heavy metal induced superoxide anions with superoxide dismutase as another strategy in E.coli lineage of Proteobacteria for protection from heavy metal toxicity (Geslin, 2001; Haferburg and Kothe, 2007). Increased levels of expression of genes encoding efflux proteins have been observed when P. aeruginosa without any previous exposure to heavy metals was subjected to heavy metal shock (Teitzel, et al., 2006; Martinez, et al., 2009). In Eloor, the diversity of Firmicutes in soil sample was restricted to B.cereus, B. filicolonicus, B. pumilus GC-Sub gpB, S.cohini, S. aureus and S.gallinarum whereas the unpolluted samples collected from Vypin consisted of 17 species of cultivable Firmicutes which included 12 species of Bacillus 3 species of Staphylococcus and 1 species each of Exignobacterium and Paenibacillus. Bacillus and Streptomyces species also possess the capacity to adsorb high amounts of metals.
from solution using polysaccharides (Vijayaraghavan and Yun, 2008; Haferburg, et al., 2009). Bacillus listeria and Staphylococcus survive in the presence of elevated levels of cadmium by employing efflux “pumping” detoxification mechanism facilitated by efflux ATPases (Gadd and Griffiths, 1978). It could be hypothesized that the proteobacteria with high diversity of metal resistance strategies survived in sediment samples collected from Eloor, the most polluted area. However, higher concentrations of heavy metals inhibit the growth of other marine bacteria and hence, can impair the homeostasis of aquatic microbial communities. Although the adverse effects of different metals on soil microbial communities have been reported (Said and Lewis, 1991; Khan and Scullion, 2002) it is difficult to evaluate the effect of multiple complex metal-mixtures on microbial communities.

5.4 Effect of AgNPs on MDR pathogenic MRB

Silver nanoparticles have emerged as an alternative therapy to control the multiplication of multiple antibiotic resistant bacteria as silver and its compounds have strong inhibitory and bactericidal effects on bacteria, fungi, and virus (Franke et al., 2001; Morones et al., 2005; Lok et al., 2006; Lara et al., 2010; Liet al., 2010; Cho et al., 2008; Silver., 2003) but lower toxicity to mammalian cells (Zhao and Stevens 1998). An important feature of nanoparticles is that, on a mass basis, more atoms are available at the particle’s surface to interact with its surroundings. At this scale, unique physicochemical characteristics appear, and the reactivity is largely increased in comparison to the nanoparticles’ bulk counterparts (Luoma, 2008). As compared to the antibiotics, the bacterial resistance against ionic silver has been observed only rarely and does not constitute any significant implications (Silver 2003). Studies on the effect of AgNPs on marine MDR pathogenic bacteria are not available in literature. MDR pathogenic MRB of the CE was inhibited by AgNPs except S. aureus. The level of susceptibility of MDR pathogens differed with the concentration of AgNP. Studies have shown that AgNPs have an antimicrobial effect on clinical E. Coli, V. cholera, P. aeruginosa, S. aureus, S. typhus and the multiple drug resistant P. aeruginosa, the ampicillin resistant E. Coli and the erythromycin resistant S. pyogenes (Lara et al., 2010; Shrivastava et al., 2007; Morones et al., 2005). AgNPs inhibitory property can be attributed to the higher surface area per unit volume and
subsequent enhancement in surface reactivity (Luoma, 2008; Ji et al., 2007), which provides better contact area on microorganisms to penetrate the cell membrane of the cells below the size range of 10 nm (Sondi and Salopek-Sondi, 2004; Xu et al., 2004). Even though the antimicrobial properties of AgNPs are receiving greater attention, the mechanism by which they kill microorganism is not well understood (Choi and Hu, 2008; Morones et al., 2005; Nel et al., 2006; Pal et al., 2007). It was evident from the SEM images in the present study that morphology of the gram negative isolates has changed after AgNPs treatment with severe damage to *E. coli* and *P. aeruginosa*. Microscopic observations of the treated MDR cells showed distinct morphological changes in cell shape or morphology of the sensitive MDR pathogenic MRB. This was similar to the morphological changes observed in *E. coli* by Jung et al., (2008). Recent electron microscopy studies have revealed that majority of AgNPs were localized in the membranes of treated *E. coli* cells (Sondi and Salopek-Sondi, 2004). SDS assay showed that the cell wall integrity of all the strains except *S. aureus* was reduced drastically after 120 minutes of incubation with AgNPs. After 80 minutes of incubation *V. alginolyticus, P. aeruginosa* and *E. coli* lost their cell wall integrity. Possible mechanisms of AgNPs interaction with cell wall can be that AgNPs attach to the cell membranes, causing changes in membrane permeability and redox cycle in the cytosol (Lok et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004). The smaller and uncharged Ag nanoparticles with higher surface areas could interfere with cell membrane function by directly reacting with cell membrane to allow a large number of the Ag atoms to attack or easily enter the cells (Nel et al., 2006; Morones et al., 2005). Intracellular fatty acids are of particular importance in the maintenance of a number of biological processes. The phospholipid portion of the bacterial membrane may also be the site of action for the silver species (Lok et al., 2006). In response to stress the total fatty acid content in bacteria may decrease or increase (Guckert et al., 1986; Guerzoni et al., 2001). An earlier report (Chattopadhyay and Jagannadham, 2003) has shown nearly exclusive role of branched fatty acids for adaptations towards environmental toxicity where the ratio of anteiso/iso fatty acid was used as one of the most important determinant for bacterial cell membrane fluidity and consequently their adaptation towards environmental stress. Since the MDR isolates were also multiple
metal resistant, it is expected that the isolates have changed fatty acid composition for tolerating heavy metals. Interestingly, there was change in fatty acid composition to AgNPs. The response of sensitive MDR pathogenic MRB was different. In the case of gram negative *E. coli* and *Vibrio sp* composition of short chain fatty acid (12:00 and 14:00) was more. In the case of Gram positive multiple resistant *B. subtilis* there was over production of branched fatty acids ie 17:0 and 15:0 iso-fatty acid to combat stress condition which is in agreement with previously published report on resistant *B. subtilis* (Hosonoand Hahn, 1986). In case of *B. subtilis* an increase in anteiso/iso ratio was observed during its growth at extremely low temperatures (Klein et al., 1999). It is paradoxical, why sensitive MDR *B. subtilis* MRB produced more of the branched fatty acid. Studies have shown that the first site of action of AgNPs is the cell wall where it induces a chain of reactions including the expression of a number of envelope proteins (OmpA, OmpC, OmpF, OppA, and MetQ) and disruption of the barrier components (Lok, et al., 2006). It appears that AgNPs unique property of higher surface area per unit volume and subsequent enhancement in surface reactivity would have induced over production of certain fatty acids which would have caused instability to the membrane and affected barrier components. However, further study is required to substantiate this hypothesis. Considering that the bacterial plasma membrane is the site of active transport, respiratory chain components, energy transducing systems, membrane stages in the biosynthesis of phospholipids, peptidoglycan, LPS and capsular polysaccharides, and the anchoring for DNA, an alteration of the membrane’s integrity would have a great impact on sensitive bacteria. It was observed that the metabolic activity of the four sensitive MDR was affected on exposure to 20 µg ml\(^{-1}\) concentration of AgNPs. AgNPs also induced DNA damage. It was high in *E. coli* and *P. aeruginosa*, wherein more than 45 and 65 % cells were affected. The extent of DNA damage was less in *B. subtilis* and *V. alginolyticus* (ca 15%). AgNPs are reported to follow the same mode of action as other silver derivatives but not that of antibiotics (β-lactamics, quinolones, aminoglycosides, trimethoprim-sulfamethoxazole, and vancomycin) mode of action. Silver ions are known to bind to sulfhydryl groups, which lead to protein denaturation by the reduction of disulfide bonds (S–S → S–H + H–S) Besides, silver ions can complex
with electron donor groups containing sulfur, oxygen, or nitrogen that are normally present as thiols or phosphates on amino acids and nucleic acids (McDonnell, 1999). Storz et al., (1990) proposed that oxygen associates with silver and reacts with the sulfhydryl (–S–H) groups on cell wall to form R–S–S–R bonds thereby blocking respiration and causing death of cells. The mechanism by which AgNPs kills MDR pathogenic MRB may be as follows. AgNPs pass through trans membrane porins (typical internal pore size in nm) for transport across cell membranes to cause the damage of cellular constituents and metabolism. Inside the cytoplasm AgNPs form low molecular weight regions (Li et al., 2010) which interfere with respiratory chain reaction (Schreuers and Rosenberg, 1982; Dibrov et al., 2002), nucleic acid stability (Ghandour et al., 1994; Fenget al., 2000; Lok et al., 2006), cell division and finally leading to cell death (Rai et al., 2009; Li et al., 2010). Resistant strains have not been reported against AgNPs until now, as AgNPs have different plasmon resonance and scattering properties at nanoscale (Merchan et al., 2006; Pandian Panacek et al., 2006; Lechiguerra et al., 2005; Srivastava et al., 2007; Yoon et al., 2008; Ayala-Nunez et al., 2009; Rai et al., 2009). MDR \textit{S. aureus} was resistant to AgNPs up to 100 µg ml\(^{-1}\). Resistance towards organic solvents and aromatic compounds is associated with alteration of bacterial cell morphology and composition of total cellular fatty acid (Isken and De Bont 1998; Sardessai and Bhosle 2002; Nair et al., 2005; Zahir et al., 2006). SDS assay showed the cell wall of \textit{S. aureus} was highly resistant to AgNPs and maintained more than 75% integrity after 120 minutes of incubation in 100 µg ml\(^{-1}\) AgNPs. Jung et al., (2008) suggested that the thickness of the peptidoglycan layer of Gram-positive bacteria may prevent to some extent the action of the silver ions. Higher inhibitory activity of AgNPs was more in \textit{E. coli} than \textit{S. aureus}. Stress conditions can regulate the membrane fluidity and stability by adjustments of membrane lipid or fatty acid composition (Navarillo et al., 1993). There was increase in 17:0 and 15:0 iso-fatty acid in the resistant \textit{S. aureus}. This is in agreement with the report that gram positive bacteria is more tolerant due to the over production of branded fatty acids during metal stress conditions as previously reported (Hosono, 1982; Hazel and Williams 1990; Pennanen et al., 1996). Since \textit{S. aureus} maintained more than 75% of integrity after 120 minutes of incubation in 100 µg ml\(^{-1}\) AgNPs, it
appears that AgNPs failed to bind and disturb the bacterial cell membrane activity (Sondi and Salopek-Sondi, 2004). Hence on exposure to 20 µg/ml concentration of AgNPs, the metabolic activity was not affected S. aureus. Interestingly, no significant changes in the metabolic activity of S. aureus were observed even at the highest (100 µg/ml) concentration of AgNPs. Genetic material of MDR cell of S. aureus was not affected by AgNPs as most of the cells (98%) were under low or no damage class in comet assay. The role of efflux pumps in rendering resistance to S. aureus to AgNPs cannot be ruled out as studies have shown that efflux pumps responsible for resistance in number of bacteria (Lix et al., 1998; Isken and De Bont, 2000; Tokunaga et al., 2004). Efflux pumps are recognized as the active systems in both gram negative and positive bacteria, which render them resistance against antibiotics and metals (Chuanchuen, 2001; Martinez, 2009; McMurry, 1980; Paulson, 2003; Sanchez, 2005; Zgurskaya, 2000). It was interesting to note that more than 60% of S. aureus cells treated with AgNPs and Verapamil incurred damage to DNA. Verapamil, derivative of phenylalkylamine has been reported as an inhibitor of several bacterial ABC efflux pumps including LmrA and calcium channel antagonists (Poelarends et al., 2002, Zechini and Versace, 2009, Kannan et al., 2009). Bacterial ABC transporters have been assigned for the translocation of nutrients across the cell membrane and the same can be shared for conferring resistance towards toxic compounds (Locher et al., 2002). It can be postulated that the efflux pump of S. aureus is versatile enough to protect it from deleterious effect of AgNPs.

This study on the metal microbe interaction in the CE provided important insights on the abundance, diversity, metabolic functions of metal resistant bacteria. The urban discharge has resulted in the increase of autochthonous and allothonous resistant Proteobacteria and Furmicutes. Most of the MRB were tolerant to multiple metal ions and functioned as an agent in the proliferation of antibiotic resistance. The mode of resistance of MRB was the efflux system. The study on the effect of AgNPs on MDR pathogenic MRB showed that AgNPs is an alternative and effective antibacterial agent. However resistance of MDR S. aureus to AgNPs points to the necessity for imparting control over the wide spread applications of AgNPs.

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