STUDIES ON METAL RESISTANT BACTERIA IN COCHIN ESTUARY AND ITS RESPONSE TOWARDS ANTIBIOTICS AND SILVER NANOPARTICLES

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in

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Under the Faculty of Marine Sciences

By

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February 2014

Dedicated to my dear parents and teachers

Certificate

*This is to certify that the research work presented in this thesis entitled "**STUDIES ON METAL RESISTANT BACTERIA IN COCHIN ESTUARY AND ITS RESPONSE TOWARDS ANTIBIOTICS AND SILVER NANOPARTICLES**" is based on the original work done by Ms. Jiya Jose (Reg. No. 3876), under my supervision at CSIR-National Institute of Oceanography, Regional Centre, Kochi, 682018, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Faculty of Marine Sciences, Cochin University of Science and Technology, Kochi, 682018 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes.*

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Declaration

*The research work presented in this thesis entitled "**STUDIES ON METAL RESISTANT BACTERIA IN COCHIN ESTUARY AND ITS RESPONSE TOWARDS ANTIBIOTICS AND SILVER NANOPARTICLES**" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, is a bonafide record of the research work done by me under the supervision of Dr. ShantaAchuthankutty, Chief Scientist, National Institute of Oceanography, Regional Centre, Kochi, 682018. No part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes*

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Contents

Chapter 1
INTRODUCTION

Metals play a significant role in all major life processes, which include maintenance of osmotic balance, stabilization of cellular organelles, electron donors and acceptors in many processes and cofactor for metalloenzymes (Gadd, 2010; Jensen, 2013; Vašková et al., 2012). Heavy metals have a density of >5 and there are 40 known heavy metals. Most of the heavy metals have essential biological functions except arsenic, cadmium, mercury, and lead (Gadd,1992). They are transported across the cell membrane through energy independent or dependent processes to participate in cellular processes. In the cell, prokaryotes including eubacteria and archaea are able to either oxidize Mn(II), Fe(II), Co(II), Cu(I), or reduce Mn(IV), Fe(III), Co(III), on a large scale and conserve energy in these reactions. Some microbes may reduce metal ions such as Hg^{2+} or Ag^{+} to Hg and Ag, but do not conserve energy from these reactions (Beal et al., 2009; Du Laing et al., 2009; Summers and Sugarman, 1974). Although many of them are essential for the growth of microorganisms, high concentration of metal in the cytoplasm is reported to have comprehensively toxic effects at cellular and sub-cellular level as metal denature protein molecules (Bierkens, 2000; Hamza-Chaffai et al., 1997). Being ancient and ubiquitous on earth, microbes have acquired a spectrum of resistance strategies compared to higher life forms depending on the environment (Rajbanshi, 2009; Rajkumar et al., 2010; Sun et al., 2010).

In marine environment, metal tolerance is generally high among microorganisms due to their reduced membrane permeability caused by the higher salt concentrations of marine water (Sprague, 1964) compared to the fresh water. Nonetheless, in marine environment, the deleterious effects of heavy metals on the biota are also long lasting as they are not easily or rapidly eliminated from these ecosystems by natural degrading processes. These metals tend to accumulate in sediment, move up the aquatic food chain and ultimately reach the human being

(Förstner et al., 1979;Nakayama et al.,2010; Peralta-Videa et al., 2009; Zhuang et al., 2009). Toxicities depend mainly on their concentration and bioavailability in the system, which in turn depends on the source, speciation, and transformation; control exerted by major sediment components to which metals are preferentially bound; competition between metals for uptake sites in organisms and the influence of environmental parameters like turbulence, salinity and pH or redox on these processes (Bryan and Langston, 1992).

The selective pressure on microbial community by heavy metal pollution leads to the emergence of resistant strains (Gadd, 2010; Hall, 2002; Hassen et al., 1998), commonly referred to as metal resistant bacteria (**MRB**). Several microorganisms have acquired metal resistance strategies which include exclusion of the metal by a permeability barrier or active export, physical sequestration and transformation and detoxification (Kisand et al., 2012; Molina et al., 2011; Pavel et al., 2013). Among the different strategy, efflux pump is the most common mechanism for adjusting the intracellular concentrations of heavy metals in microorganisms (Deb et al., 2013; Hajdu et al., 2010;Yamane et al., 2007). The ascribed function of the efflux pumps is to regulate the transportation of metabolic products, lipids, sterols, sugars etc. across the cell membrane (Borges et al., 2003; Borst et al., 2000; Tsuji et al., 1992). The same efflux pump mechanism against Cu, Co, Zn, Cd, Ni and As is also shared for providing resistance against tetracycline, chloramphenicol and β-lactam antibiotics (Baker-Austin et al., 2006).An unfortunate side effect of the heavy metal resistance is that the same genetic platform can be shared against antibiotics leading to horizontal transfer of these genes between clinical isolates in the environment and indigenous environmental isolates. Recently silver nanoparticles (AgNPs) has emerged as a promising antimicrobial compound against Gram negative and positive nosocosmial microorganisms and HIV, hepatitis B virus, respiratory syncytial virus, herpes simplex virus and monkey pox virus (Ayala-Nunez et al., 2009). Resistance to antimicrobial silver nanoparticles and its mode of action are all hitherto unknown in the environment.

The increasing trend in concentration of heavy metals in the environment has attracted considerable attention amongst ecologists globally during the last six decades

and has also begun to cause concern in most of the major metropolitan cities. The direct impact of heavy metal pollution on microbial ecosystem includes the alterations in the physiology, diversity and abundance. In general, microorganisms from heavy metal polluted environment utilize majority of the energy for cell survival and as a consequence, their contribution to the ecosystem functioning become meager. Polymeric and particulate organic matter are processed by the hydrolytic enzymes secreted by microorganisms before its transportation across the cell membrane (Bong et al., 2010; Chrost and Rai, 1994). The selective pressure of heavy metal pollution leads to apparent reduction in the extracellular enzyme activity of that particular ecosystem (D'Souza et al., 2006;Lasat, 2002; Li et al., 2006; McGrath et al., 2001; Silver, 1984 ; Wang et al., 2007), which indirectly affects the microbial loop and the ocean productivity (Bong et al., 2010; Chakravarty and Banerjee, 2008; Haferburg and Kothe, 2007; Hoostal et al., 2008). Heavy metals also contribute indirectly to global warming. They inhibit bacterial nitrous oxide reductase enzyme which may lead to the accumulation of potent green house gas N_2O (Dickinson and Cecerone, 1986; Haferburg and Kothe, 2012; Sobolev and Begonia, 2008). Consequently, at the end of each time period, the pollution problem takes menacing concern.

Estuaries are ecologically and economically important ecosystems with one end opening to the ocean and the other end receiving the fresh water. Apart from the natural processes, estuaries receive additional dosage of heavy metals through industrial and domestic discharges (Balachandran et al.,2002, 2006, 2008; Nair et al., 2006; Noah and Oomori, 2006). The processes in estuaries are dictated by tidal variations and freshwater influx, which directly influence the salinity and bioavailability of heavy metals in the estuary. The heavy metals reaching the estuaries through fresh water may get trapped in the sediment and their transportation towards the marine environment may take a long time depending on the water currents. Therefore, heavy metal pollution and bioaccumulation may have significant effect on the health of the estuaries and the inhabitants than those in the sea to which the estuaries open. In Indian estuaries, the heavy metal concentrations in sediment/ water and in community are much higher than the permissible limits ascribed by international standards (Agadi et al., 1978; Alagarsamy 2006; Ramkumar et al., 2012;

Vardanyan and Ingole, 2006). Consequently, the effect of heavy metal pollution on the diversity and dynamics of microorganisms in the marine environment is a topic of growing environmental concern as it can provoke unintended alterations in the functioning of marine ecosystems directly or indirectly. The effects of heavy metals on macro-organisms are often apparent and well-documented from Indian waters but its effects on microbial communities have received less attention. There are limited and scattered studies on metal-microbe interaction. Few studies are on the level of tolerance and biotransformation of metals in bacteria isolated from coastal waters, estuaries and from deep sea (Nair et al., 1993; Krishnan et al., 2007; Sujith et al., 2010 and 2014; Ramaiah and De, 2003). There is an isolated report on the distribution of Hg resistant bacteria along the coastal water of India (De et al., 2008). Nair et al., (1992) and Chandy, (1999) have reported that heavy metal resistance was more pronounced among gram-negative bacteria and that metal and antibiotics resistance are linked to chromogenesis. Nithya et al., (2011) have studied the diversity of metal resistant bacteria and its association with antibiotics (Nithya and Pandian, 2010) in the Palk Bay sediments. These few studies clearly show that there is a dearth of information on metal - microbe interaction from Indian waters.

The Cochin estuary (CE), which is one of the largest wetland ecosystems, extends from Thanneermukkam bund in the south to Azhikode in the north. It functions as an effluent repository for more than 240 industries, the characteristics of which includes fertilizer, pesticide, radioactive mineral processing, chemical and allied industries, petroleum refining and heavy metal processing industries (Thyagarajan, 2004). Studies in the CE have been mostly on the spatial and temporal variations in the physical, chemical and biological characteristics of the estuary (Balachandran et al., 2006; Madhu et al., 2007; Menon et al., 2000; Qasim 2003;Qasim and Gopinathan 1969) . Although several monitoring programs have been initiated in the CE to understand the level of heavy metal pollution, these were restricted to trace metals distribution (Balachandran et al., 2005) or the influence of anthropogenic inputs on the benthos and phytoplankton (Madhu et al., 2007;Jayaraj, 2006). Recently, few studies were carried out on microbial ecology in the CE(Thottathil et al 2008a and b;Parvathi et al., 2009and 2011; Thomas et al., 2006;

Chandran and Hatha, 2003). However, studies on metal - microbe interaction are hitherto not undertaken in this estuary. Hence, a study was undertaken at 3 sites with different level of heavy metal concentration tounderstand the abundance, diversity and mechanisms of resistance in metal resistant bacteria and its impact on the nutrient regeneration. The present work has also focused on the response of heavy metal resistant bacteria towards antibacterial agent's antibiotics and silver nanoparticles. Accordingly, the following objectives have been proposed for the thesis:

1. Distribution and enzyme expression profile of metal resistant bacteria along a pollution gradient in the Cochin Estuary.
2. Differential response of metal resistant bacteria towards antimicrobial agents.
3. Mechanisms of microbial resistance/sensitivity towards silver nanoparticles.

Chapter 2
REVIEW OF LITERATURE

Contents

This chapter covers all the relevant literature that are dealing with the importance of bacteria and metals, toxic effects of metals on bacteria, its modes of resistance to heavy metals, heavy metal pollution and ecological significance of metal-microbe interaction in marine environment. The review of literature has been grouped and sub-titled according to the various aspects that have been studied.

2.1 Metal –Microbe interaction

2.1.1 Introduction

Microorganisms are ubiquitous and play a significant and dominating role in the cycling of organic and inorganic matter in marine ecosystem (Ammerman and Azam, 1991; Chrost.,1991; Chrost and Overbeck,1987). They are efficient degraders as they can penetrate the water and sediment most intimately by their high number and size (Hoppe and Gocke, 1993). They possess a variety of catabolic enzymes which allow them to decompose various dissolved and particulate substrates (Meyer-Reil., 1994) thereby making it available for other organisms through the 'microbial loop' (Azam et al., 1983). It is well recognized that microbial communities play a vital role in the biogeochemical cycles of elements in various marine environments and maintain the ecosystems' health (Pace, 1997). Further, its' high surface area to volume ratio can interact with metals in the surrounding environment (Ledin, 2000).

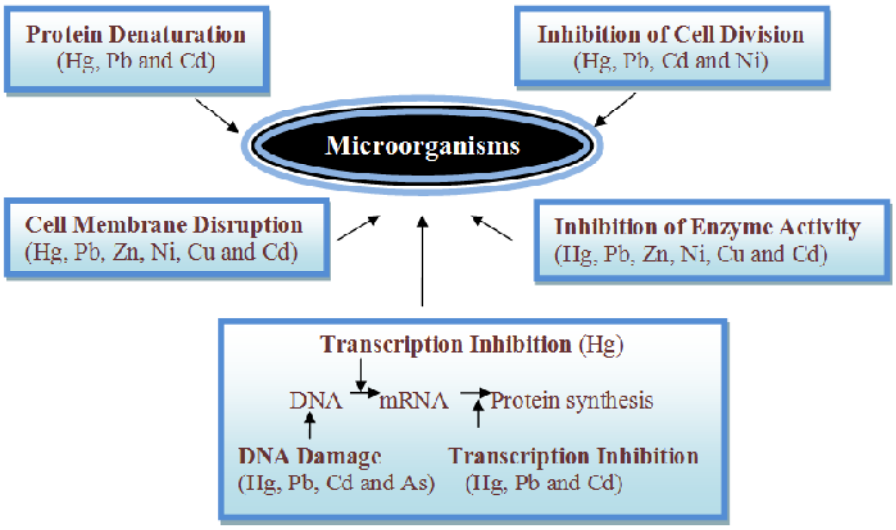
2.1.2 Role of Heavy Metals in bacteria

Metals are important constituents of the earth's crust from the time it was evolved. Heavy metals are a group of about 40 chemical elements with relatively high

density. Early life has arisen in the presence of metals and living systems have evolved to use some metals as vital constituents of its biological systems (Diaz-Ravina et al., 1994, Gadd, 1990a and 1990b, Lehninger et al, 1993). However, some metals such as arsenic, cadmium, mercury, and lead have no known essential biological function (Gadd, 1992). It serves as prosthetic groups in many proteins and dictates the configuration of the active site of enzymes. They serve as redox centers transferring electrons in important redox reactions. For example, iron participate in many biological processes, such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Wang et al., 2005). Iron intake mechanism is greatly controlled by the efficiency of iron scavenging siderophores in bacteria as predominant form of iron is extremely insoluble in water. Selenium is required for the function of selenoproteins (Gu et al., 2002). Magnesiumis also involved in the stabilization of ribosomes, nucleic acids and cell membrane. Under optimal concentrations, microorganisms transport metals across the cell membrane through energy independent or energy dependent processes (Nies, 1999; Nies and Silver, 1995; Deng and Wilson, 2001). Energy independent systems are the fast and unspecific processes guided by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. Energy dependent transport is substrate specific and slower and often uses ATP hydrolysis as the energy source. These expensive uptake systems are activated only by the cell only during the times of need, starvation or a special metabolic situation (Nies, 1999; Nies and Silver, 1995). In certain specialized ecosystems like hydrothermal vent, Mn nodule and cobalt crust areas in the ocean the concentration of metals are high. In these systems metal tolerant bacteria are dominant and the ecosystem function is metal based (Jeanthonand Prieur, 1990, Rathgeber et al., 2002, Vetriani et al., 2005, Lianos et al., 2000; Duxbury and Bicknell,1983). Since microbial encounter with metals are unavoidable in the environment, it is not surprising that microbes (chemolithotrophs) 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).

2.1.3 Toxic effect of heavy metals on bacteria

A feature of heavy metal physiology is that even though many of them are essential for microbial growth, they are also reported to have comprehensively toxic effects on cells when present in slightly higher concentration than the required (Gadd and Griffiths, 1977). Elevated concentrations of metal ions may interrupt the normal functioning of energy independent systems resulting in an "open gate" condition leading to the accumulation of metals in cytoplasm. Zinc a micronutrient at elevated concentrations can induce DNA damage, cell membrane perturbations and oxygen stress (Shen et al., 1999). Cadmium ions are highly toxic to cells at very low concentrations. Cadmium is taken up through calcium channels of the plasma membrane, and accumulates intra-cellularly due to its binding to cytoplasmic and nuclear material and inhibits the biosyntheses of DNA, RNA and protein, induce lipid peroxidation and cause chromosome aberrations (Beyersmann and Hechtenberg, 1997). Some metals (Hg^{2+}, Cd^{2+}, and Ag^{+}) tend to bind to SH groups of proteins and inactivate their enzyme functions. Other heavy-metal cations may interact with physiological ions, Cd^{2+} with Zn^{2+} or Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, Zn with Mg^{2+}, thereby inhibiting the functioning of the respective physiological cation (Nies and Silver, 1995). It may bind to glutathione in Gram negative bacteria and the resulting bisglutathionato complexes tend to react with molecular oxygen to form oxidized bisglutathione (GS-SG), the metal cations and hydrogen peroxide (Kachur et al., 1998; Nies and Silver, 1995). Since the oxidized bisglutathione has to be reduced again in an NADPH-dependent reaction and the metal cations immediately bind to another two glutathione molecules, heavy-metal cations cause a considerable oxidative stress. Heavy metal oxyanions interfere with the metabolism of the structurally related non metal and reduction of heavy metal oxyanion, leading to the production of radicals. The figure summarizes the toxic effect of heavy metals on microorganisms (Gadd 1992).

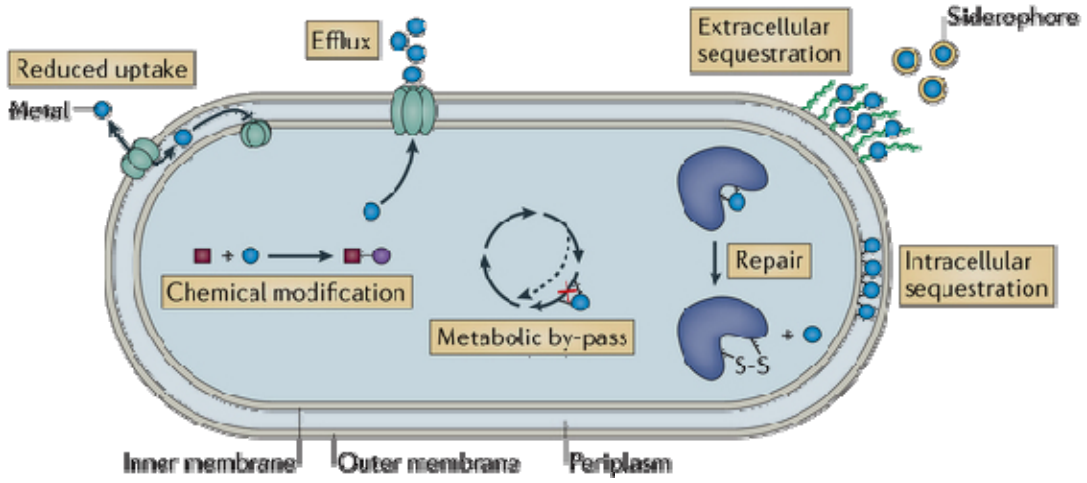


Effect of heavy metal on microorganisms

2.1.4 Mode of resistance in bacteria

Microorganisms adopts various mechanisms to combat metal toxicity such as metal sorption, mineralization, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell (Mergeay et al., 2003; Mata et al 2000). Discerning which mechanism is involved in a bacterial community is made difficult by the multitude of resistance pathways utilized by bacteria (Silver, 1996; Alonso et al., 2001). An important protective device used by many microorganisms involves sequestration of toxic metals by cell-surface moieties. Metallothioneins comprise a group of small, cysteine-rich proteins with high affinity and selectivity for heavy metals such as cadmium, copper and zinc. Bacteria expressing metallothionein genes have been shown to accumulate copper, cadmium and other heavy metals effectively (Pazirandeh et al., 1995). Another resistant mechanism is by metal ion sorption in microorganisms (Stockdale et al.,2010). The structure of cell wall affects both the rate and extent of metal sorption. Glycoproteins present on the outer cell wall side of Gram-positive bacteria have been suggested to have more potential binding sites for metal ions than the phospholipids and LPS and hence are responsible for the observed difference in capacity (Ghandour et al.,1988). Mechanisms of metal resistance in microbes also include precipitation of metals as phosphates, carbonates and/or sulfides and volatilization via methylation or ethylation(Gadd, 1990; Silver, 1996). Most cells use two types of uptake system for heavy-metal ions: one is fast, unspecific and the other is slow , specific (Nies and

Silver, 1995). Another well studied resistant mechanism is efflux pumps seen as the active system in both Gram negative and positive bacteria, which render them resistance against metals (Chuanchuen et al.,2001; Martinez, 2009; McMurry et al, 1980; Paulson et al., 2000; Sanchez , 2005; Zgurskaya and Nikaido, 2000). The efflux pumps are membranes associated with active transporters, which are reported initially in Gram negative bacteria as a means to remove antibiotics from the cytoplasm (McMurry et al, 1980; Parkhill et al 2001). Genome sequence analysis reveal that, on average, efflux pumps constitute at least 10 % of the transporters in several bacterial species and they usually have the capability of extruding a broad range of structurally different compounds (Paulson et al., 2003). Studies have demonstrated that the efflux pumps of microorganisms are capable of extruding heavy metals (Silver and Phung, 1996 and 2005; McMurry et al., 1980; Nies and Silver, 1995; Paulson et al , 2000; Zgurskaya Nikaido,, 2000; Nies 2000). Several bacterial strains are resistant to Ag ions (Silver, 2003) and can even accumulate Ag particles in their cell wall to as much as 25 % of the dry weight biomass. The silver resistant *Pseudomonas stutzeri* AG259 accumulates silver NPs of particle size 35 to 46 nm in their cell (Slawson et al., 1992). The defensive mechanism of these microorganisms for metal detoxification has been suggested as the biological pathway that reduces the metal ions and precipitates the metal compounds in the periplasmic space (Xie et al., 2007, Klaus et al 2001). However, the mechanism by which bacteria reduces metal ions to NPs is hitherto unknown. The different mechanisms adopted by bacteria are depicted in the figure below (Nies, 1999).



Metal resistant mechanism in bacteria

2.2 International scenario

2.2.1 Heavy metal pollution

Heavy metals are both naturally and artificially present in ecosystems. Heavy metals enter the marine environment through atmospheric and land based effluent sources. Heavy metals and trace elements are by-products of many industrial processes, contributing to varying amounts of different metals and trace elements and as such are discharged as waste into the marine environment (Robson and Neal, 1997). The oceans provide a major sink for many heavy metals and their compounds. Different degrees of heavy metal pollutions have been observed in the coastal areas of northwestern Europe, mostly attributed to industrial discharges, waste-disposal streams, and atmospheric deposition of exhaust gases. However, considerable heavy metal pollution of sediments of harbours and marinas has been attributed to the application of antifouling paints on ship hulls (Schiff et al. 2004; Warnken et al. 2004). Dredging operations can increase metal mobilization by whirling up the fine sediment particles and allowing oxygen to come in contact with previously buried and reduced sediments. The extent of metal release depends on local parameters such as sediment geochemistry, currents, grain size, pH, and salinity (Van den Berg et al., 1998). Unquestionably, a variety of biological parameters also play a role in metal mobilization. The natural cycling rates of many metals are being disturbed by anthropogenic activities, especially the release from industrial, domestic and urban effluents of increasing amounts of Pb, Zn, Cd, Hg and Cu (Schindler et al., 1991) into the estuaries and coastal waters. Reports on metal pollution and effects on marine ecosystems have been published by United Nations Environment Programme (UNEP) and GESAMP (Group of Experts on Scientific Aspects of Marine Environmental Pollution, Advisory body, established in 1969, consists of 25-30 international experts on marine pollution and emerging issues, is sponsored by UN, IMO, FAO, UNESCO-IOC, WMO, IAEA, UNIDO) and the environmental specialists (UNEP 1983, GESAMP, 1990 in Kullenberg, 1986). Goldberg (1995) reviewed different sources of heavy metal inputs into the sea and their possible role in ecosystems. These metals tend to accumulate in sediment and move up the aquatic food chain, ultimately reaching human being, in whom they produce chronic and acute ailments (Forstner et

al.,1979), including a number of DNA mutations (De Flora et al., 1994). In the last few decades there has been growing concern on metal pollution not only in marine but also in freshwater and terrestrial ecosystems. The metals considered toxic and which are of concern have been restricted largely, but not exclusively, to ten which appear to be the most poisonous to marine life. These include, in order of decreasing toxicity mercury, cadmium, silver, nickel, selenium, lead, copper, chromium, arsenic and zinc. Stringent implementation of environmental legislations by EEA has led to a reduction in the discharge of these metals during the last 15 years.

2.2.2 Ecological Studies on Metal–Microbe interaction

Microbial communities are key players involved in metal mobility (Ford and Ryan, 1995). In marine environments, the heavy metal concentration is high and marine microbes as a rule are exposed to these higher concentrations and they can tolerate higher concentration of heavy metals, five- to ten folds higher than their concentrations in sea- water. Metals have a high ecological significance due to their toxicity and accumulative behavior. Heavy metals when present in slightly higher concentration than the required concentration become toxic as a result of their ability to denature protein molecules (Gadd and Griffiths, 1977). They exert a selective pressure on Gram-positive and negative bacteria (Wuertz et al., 1997; Kozdroj and van Elsas, 2001; Abou-Shanab et al., 2007), leading to the emergence of resistant strains popularly referred to as **"metal resistant microorganisms" (MRB)**. Bacterial resistance mechanisms to—and intracellular uptake of—trace metals from seawater have been shown to follow different pathways (Robson and Neal, 1997). Many researchers have indicated that the abundance, diversity, and functions of microbial communities may be altered from their pristine conditions by the presence of metal pollutants (Baath, 1989; Chang et al., 1993; Ellis et al., 2003; Frostegard and Baath, 1996; Konopka et al., 1999; Stephen et al., 2000). It has therefore been suggested that the microbial abundance, diversity and metabolic activity in metal-contaminated environments may provide an indication of the health of the ecosystem as a whole (Kowalchuk et al.,1997; Nielsen and Winding, 2002; Stephen et al., 2000). Historically, culture-based monitoring has been the most practical and widely used method employed to observe microbial communities. Plate count techniques are being

used to evaluate bacterial abundance in contaminated environments (Diaz-Ravina and Baath, 1996; Ellis et al., 2003; Feris et al., 1999). A number of these studies have reported a loss of bacterial abundance and metabolic activity with increasing prevalence of heavy metals. It is now well accepted, though, that 1% or less of bacterial species are represented in some of these studies (Amann et al., 1995; Pace , 1997). There is sorption or chelation of metals to unspecified organic compounds found in most biological media (Chang et al., 1993; Tong and Sadowsky, 1994). Free metal ion, ordinarily considered to be the toxic metal species that ultimately determines the microbial response to the metal, rarely approaches the total metal concentration added to media (Angle and Chaney, 1989). In recent decades, the development of molecular-based techniques have allowed for a more complete analysis of both culturable and nonculturable microorganisms in contaminated environments. As a result, many recent studies have relied heavily on the use of nucleic acid-based monitoring to assess the anthropogenic effects on microbial communities (Del Val et al., 1999; Feris et al., 1999; Kuo and Genthner, 1996; Pennanen et al., 1996).

2.2.3 Metal Tolerance in Bacteria

The tolerance levels of individual isolate to different metals from different locations were carried out mostly by using amended medium (Nies, 1999,2003 and2007; Silver 1996; Surosz and Palinska, 2004). Culture studies on individual isolates using amended medium have inherent limitations. The MICs determined with traditional approach media cannot be related to actual metal concentrations in the habitat from which bacteria were isolated. In spite of these limitations, the technique of MICs remains a valid approach to evaluate the action of heavy metals on the microbial activity in polluted habitats (Mergeay et al., 1985; Liesegang et al., 1993; Taghavi et al., 1997). All the Gram-negative isolates from marine systems are far moreresistant than Gram-positive strains (Ravel et al., 1998 and 2000). The level of tolerance varied among the species and studied metals. The minimum inhibitory concentration of the metals ranged from 0.01 mM – 40 mM. The order of toxicity of the metals was found to be Hg > Cd > Co > Cr > Cu > As > Zn >Pb> Ni (Abou-Shanab et al., 2007). Nieto et al. (1989) reported that *Halobacteriummediterranei*,

from solar salterns and hypersaline soils located in Cadiz, Huelva, Alicante, Mallorca and the Canary Islands, Spain, tolerated eight different metals. Tolerance of bacteria to 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 (Silver and Phung, 1996; Vieira and Volesky 2010; Xie et al 2010).

2.2.4 Effect of metal on bacterial abundance

The abundance, diversity, and functions of microbial communities may be altered from their pristine conditions by the presence of metal pollutants (Baath et al, 1998; Chang et al., 1993; Ellis et al., 2003; Frostegard et al., 1993; Konopka et al., 1999; Stephen et al., 2000). Studies in the eighties, had shown a decrease in the soil microbial biomass C and N as a result of long-term exposure to heavy metal contamination(Knight et al., 1997), which was due to microorganisms in soil under heavy metal stress diverting energy from growth to cell maintenance functions (Killham, 1985). Soil microbial biomass, which plays an important role in nutrient cycling and ecosystem sustainability, has been found to be sensitive to increased heavy metal concentrations (Garland, 1997; Giller et al., 1998; Grayston et al., 1998; Liao and Xie, 2007; Wang et al., 2007). Further studies have also reiterated that trace metals have negative effect on the microbial biomass (Aoyama and Nagumo, 1997; Brookes and McGrath, 1984; Frostegard et al., 1993; McGrath et al., 1997), microbial diversity (Rasmussen and Sorensen, 1998) or microbial activities (Magalhães et al., 2007;Wang et al., 2005; Ross et al 2002) in some environments. However, in some cases, microbial biomass (Bååth et al., 1998 Feris et al., 1999; Knight et al., 1997;Shi et al., 2002;) bacterial diversity (Bouskill et al., 2010; Sorci et al., 1999;) and microbial activity (Barajas-Aceves, 2005; Magalhães et al., 2007) were relatively insensitive to high metal loads. It was concluded that the unaffected communities may have become tolerant due to individual acclimation, genetic or physiological adaptation and loss of sensitive species due to long-term exposure (Bouskill et al., 2010; Ogilvie and Grant, 2008). In addition, it was concluded that some of these unaffected communities were possibly protected by high concentrations of organic substances or clays which are known to complex metals and reduce their

bioavailability (Bouskill et al., 2010; Sorci et al., 1999). In certain polluted environments, communities were even more active, with higher bacterial abundances or higher diversities than the corresponding reference ecosystems (Feris et al., 1999; Bouskill et al., 2010). A few studies on metal microbes in combination with detailed metal analyses indicated localized and short-term effects of metal exposure (Diaz-Ravina and Baath 2001; Rasmussen and Soerensen 1998). Rasmussen and Sorensen (1998) noticed high levels of Hg resistance (50ppm) among the bacterial isolates from a mercury-contaminated site inside the Copenhagen Harbour. However, it is very difficult to draw general conclusions and it seems that each microbial community is unique and reacts in a different way. Studies from different sites affected by a variety of anthropogenic activities in European and North American coasts showed the occurrence of culturable heterotrophic bacteria capable of tolerating ca. 0.5 ppm Hg (Barkay, 1987; Rasmussen and Sorensen, 1998; Reyes et al., 1999). The dominance of Gram-negative MRB was observed in Antarctic waters also (De Souza et al., 2006).

2.2.5 Effect of metals on bacterial diversity

The outcome of metal pollution include a decreased bacterial biodiversity, an irreversible extinction of many sensitive species and the selection of cosmopolitan ones that are adapted better to contemporary environment.

Among the living biota, the microbial community is a suitable example where these results can be observed (Alexander, 1994; Baath, 1989; Giller et al., 1998; Van der Leila et al, 1997; Wuertz et al., 1997). The present understanding of microbes in natural environments is based on cultivation and isolation techniques. In this way, only a small fraction of the bacteria that live in marine habitat has been recognized. Recently, new techniques like nucleic acids and fatty acids signature biomarkers of diversity have been used successfully in monitoring the impact of metals on microorganisms in natural or induced bioremediation as it allowed a more complete analysis of both culturable and nonculturable microorganisms (Chang et al., 1993; Del Val et al., 1999; Feris et al., 1999; Kuo and Genthner, 1996; Pennanen et al., 1996) in contaminated environments. There is a reduction of microbial diversity in contaminated soil, and significant shifts in the community structure, leading to the dominance of only a few populations (species) and the disappearance of others, some

of which were never isolated by conventional methods (Kozdro´ja ,and Elsasb,2001). Some studies were on long-term impact of heavy metals on bacterial diversity of marine sediments (Powell et al., 2003; Sorci et al., 1999). Sorci et al., (1999) observed an increase in biodiversity whereas no measured change or the exact opposite response was recorded along with heavy metal contamination (Sandaa and Enger, 1994). These differences might be explained by adaptation time and / or co-contamination with organic material, but these assumptions remain speculative. There was substantial statistical variation between and within the control groups of several polluted and pristine Antarctic sediments (Powell et al. 2003). *Pseudomonas*sp was the most copper-tolerant bacterial strains from naturally polluted environments (Hassen et al., 1998).

2.2.6 Effect of metal on bacterial enzymes

Bacterial enzyme activity has been applied in monitoring metal polluted environment (Dutka et al., 1988). Heavy metal pollution exerts an apparent reduction in the extracellular enzyme activity which has significant impact on ecosystem processes (Lasat, 2002; Li et al., 2006; McGrath et al., 2001; Silver, 1984; De Souza et al., 2006; Wang et al., 2007;). It has been reported that both Cu- and Zn-ions act as inhibitors of urease activity. The Cu-ion, forming a more stable sulfide, is a considerably more powerful inhibitor than the Zn-ion.Many reports have shown that short-term or long-term exposure to toxic metals results in the reduction of microbial activities (Baath, 1989; Bardgett andSagger, 1994; Doelman, and Haanstra,1984 and 1986; Duxbury, 1983; Hoppe,1991; Hoppe and Gocke , 1993;Lasat, 2002; McGrath et al., 1997 and 2001; Revel et al., 1998) and in mangrove sediments (Tam and Yao, 1998). A study by Bong et al., (2010) on individual isolates showed that high concentration of Zn inhibits the aminopeptidase activity, but no change was seen for trypsin and chymotrypsin. De Souza et al. (2006) have reported a reduction in the multiple enzymes among bacteria with multiple metal resistances. This apparent reduction in the extracellular enzyme activity will have significant impact on ecosystem processes (Silver, 1984; McGrath et al., 2001; Lasat, 2002; Li et al., 2006; DeSouza et al., 2006; Wang et al., 2007).

2.2.7 Transfer of metal resistance among bacteria

Numerous studies have reported the incidence of bacterial plasmids (Cervantesand and Gutierrez-Corona,1994; Foster, 1981; Franke et al., 2001; Gadd, 1990; Haferburg et al., 2009 and 2010; Henschke and Schmidt, 1990) and its mobilization by indigenous bacteria in marine sediments and water of estuarine and pelagic ecosystems(Henschke and Schmidt, 1990; Paulson et al, 2003). Some of the heavy-metal-resistant bacteria are shown to possess specific plasmids. Marine plasmids with heavy metal resistance traits obtained either through exogenous or endogenous methods have been the ability to self-transfer and transfer itself to broad host ranges (Sandaa and Enger, 1994). The gene responsible for metal resistant efflux pumps can be transferred from one bacterium to another through mobile genetic platforms such as plasmids and transposons (Silver, 1996; Silver and Ji, 1994; Top et al., 1994). This has been shown in Epilithon MRB group (Henschke and Schmidt,1990).

2.2.8 Co-occurrence of metal and antibiotic resistance in bacteria

Co-resistance is a potential mechanism of dual resistance that occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron (Chapman, 2003). Recently, some insight has also been gained into the genetic flexibility of mixed microbial populations with respect to metal contamination and widespread occurrence of lateral gene transfer in response to metal toxicity (Cook et al., 1972; Sobecky, 1999). Bacteria resistant to both antibiotics and heavy metals are also isolated from non-polluted environment with greater abundance from polluted sites of Colgate Creek in Baltimore Harbour. Nakagawa et al. (2005a and 2005b) suggested that the combined expressions of antibiotic resistance and metal tolerance may not be a fortuitous phenomenon but rather is caused by selection resulting from metals present in an environment. Studies have shown the occurrence of metal tolerance and antibiotic resistance in bacteria (Oyetibo et al., 2010;Timoney et al., 1978). Berg et al., (2005) found Cu resistant isolates had a higher incidence of antibiotic resistance as compared to copper sensitive isolates, indicating that these metal and antibiotic traits are associated. Sabry et al., (1997) found the highest incidence of metal-

antibiotic double resistance existed between lead and all antibiotics (100%), copper and penicillin (95%) and nickel and ampicillin (83.3%). and the role of plasmids in conferring resistance to both antibiotics and metals has been demonstrated (Foster 1981; Lyon and Skurray 1987). 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 β-lactams (Baker-Austin et al., 2006). Jacoby (1974) found that R-plasmids determining gentamicin resistance also determine a number of other properties such as resistance to ultraviolet (UV) light, inorganic and organic Hg compounds. The table below gives the list of shared structural and functional characteristics of prokaryotic antibiotic- and metal resistance systems.

Resistance mechanism	Metal ions	Antibiotics[a]	References#
Reduction in permeability (b)	As, Cu, Zn, Mn, Co, Ag	Cip, Tet, Chlor, ß-lactam	Silver, S. (1996), Ruiz, N. (2003)
Drug and metal alteration (c)	As, Hg	ß-lactams, Chlor	[Mukhopadhyay and Rosen (2002), Wright, (2005)
Drug and metal efflux (d)	Cu, Co, Zn, Cd, Ni, As	Tet, Chlor, ß-lactams	Nies, D.H. (2003), Levy (2002)
Alteration of cellular target(s) (e)	Hg, Zn, Cu	Cip ß-lactams, Trim, Rif	Barkay et al. (2003), Roberts, (2005)
Drug and metal sequestration (f)	Zn, Cd, Cu	CouA	Bontidean, et al. (2000) Castillo, et al. (1991)

a) Abbreviations: Chlor, chloramphenicol; Cip, ciprofloxacin; CouA, coumermycin A; Rif, rifampicin; Tet, tetracycline; Trim, trimethoprim.

b) Includes reduction of membrane permeability to metals and antibiotics.

c) Includes drug and metal inactivation and modification.

d) Includes rapid efflux of the metal and antibiotic.

e) Includes alteration of a cellular component to lower its sensitivity to the toxic metal and antibiotic.

f) Includes drug and metal sequestration.

References in Baker-austin, 2006)

All these studies support the hypothesis that metal exposure results in increased frequency of antibiotic tolerance in bacteria. This was confirmed using culture-independent method in a microcosm by Stepanauskas et al., (2006) and is not limited to a subset of metals or antibiotics.

Microorganisms resistant to both antibiotics and metals have been isolated from metal-contaminated environments such as estuaries (Allen et al., 1977, Timoney

et al., 1978), soils(Baker-Austin et al., 2006; De Souza et al., 2006; McArthur and Tuckfield, 2000) sewage (Varma et al., 1976) in 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). Occurrences of multiple co-resistances to metal and antibiotics among bacteria has become a global phenomena as it was observed among bacteria from Antarctic (De'Souza et al., 2006) as well as from rivers and bays (Traxler and Wood, 1981). The presence of metal and/or antibiotic-resistant bacteria in natural habitats is now well established (Austin et al.,1977; Lulietal.1983). Numerous studies have reported the occurrence of bacterial plasmids and mediation of resistance in marine sediments and estuarine and pelagic ecosystems (Cervantes and Gutierrez, 1994; Foster, 1983; Franke, 2001; Gadd, 1990; Haferburg et al, 2009 and 2010; Henschke and Schmidt, 1990).

2.2.9 Effect of Silver Nanoparticles (AgNPs) on bacteria

Silver and its compounds are well known for their antibacterial property, since the age of ancient Egyptians (Klasen, 2000; Kvtek et al., 2008) and the most widely used silver preparation in human medicine, sulfadiazine, was introduced in 1960s (Kvtek et al., 2008). Currently, silver sulfadiazine is listed by the World Health Organization as an essential anti-infective topical medicine. AgNPs small size and larger surface area demonstrated greater bactericidal efficiency (Baker, 2005; Sarkar et al., 2007). In comparison to the antibiotics, the bacterial resistance against ionic silver has been observed only rarely and does not constitute any significant implications (Silver, 2003). 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 including *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 *Streptococcus pyogenes* (Ayala-Nunez et al., 2009; Choi, 2008; Sondi and Salopek-Sondi, 2004 ; Shrivastava et al., 2007;), fungi (Ghandour et al., 1988), and virus (Frankeet al., 2001; Merchan, et al. 2006; Morones et al., 2005; Li et al., 2010; Lara et al., 2010; Lok et al., 2006; Choi et al., 2008; Silver., 2003) but lower toxicity to mammalian cells (Zhao and Stevens, 1998).

2.2.10 Mode of inhibition of AgNPs

Even though the antimicrobial properties of Ag NPs are receiving much attention in healthcare, the mechanism by which they kill microorganism is not well defined (Morones et al., 2005; Nel et al., 2006;). Gogoi et al., (2006) demonstrated that silver nanoparticles have no direct effect on either cellular DNA or protein. But scanning electron microscopic studies showed that Nano Particles (<10 nm) may enter the cell directly to inhibit microbial growth (Morones et al., 2005); change the membrane permeability, collapse the proton motive force, and redox cycle in the cytosol (Lok et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004) or disrupt the mitochondrial respiratory chain leading the production of ROS even without UV light and interruption of ATP synthesis, which in turn cause DNA damage Electron dense areas were observed inside the cell of bacteria treated with silver ions, while a large number of nanoparticles were observed in the case of nanoparticle treated ones (Morones et al., 2005). Amro et al., (2000) suggested that metal depletion may cause the formation of irregularly shaped pits in the outer membrane and change phospholipid portion of the bacterial membrane and permeability by progressive release of lipopolysaccharide molecules and membrane proteins (ompA, ompC, ompF, oppA and MetQ) (Lok et al., 2006). While both *E. coli* and *S. aureus* depict higher sensitivity to the silver nanoparticles compared to the copper nanoparticles, the difference is less for *S. aureus* compared to *E. coli*. No strain specificity were reported by Panacek et al., (2009) for silver nanoparticles on two strains of *S. aureus* as Ag+ can lead to enzyme inactivation via formatting silver complexes with electron donors containing sulfur, oxygen, or nitrogenor by displacing native metal cations from their usual binding sites in enzymes (Ghandour et al.,1988)

Industrial development has resulted in severe disturbance of ecological balance in most ecosystems. The outcome of the disturbance include a decreased biodiversity, an irreversible extinction of many sensitive species and the selection of cosmopolitan ones that are adapted better to contemporary environment. All the above studies have shown that MRB are ubiquitous and depend on various factors including the concentration and bioavailability of heavy metals in the microenvironment, abundance and diversity of the natural population as well as drug resistant ability of the bacteria

(Bong et al., 2010; Geslin, et al., 2001; Haferburg and Kothe, 2007). The common mechanism adopted for resistance to toxic compounds isthe ABC transporters (efflux) which transports nutrients across the bacterial cell membrane (Locher et al., 2002). This property can be beneficially used for metal bioremediation. The significance of metal-microbe study in metal-contaminated environments may provide an indication of ecosystem health as a whole (Ellis et al., 2003; Kowalchuk et al., 1997; Nielsen and Winding, 2002; Stephan et al., 2000).

2.3 National Scenario

2.3.1 Heavy Metal Pollution

The concentration of heavy metals is low in the Arabian Sea, except for a few localized areas (Kureishyet al., 1986; Sanzgiriet at., 1988 whereas the concentration of heavy metals in the coastal and impacted Indian estuaries are higher (Zingade et al., 1976,1988, Subramanian et al., 1988, Mukarje and Kumar 2012, Kumar and Edward 2008, Banarjee and Gupta 2012) than the permissible limits ascribed by international standards ((Agadi et al., 1978; Alagarsamy, 2006; Ramkumar et al., 2012; Vardanyan and Ingole, 2006; Senthilnathan et al 1998). The geochemistry of sediments of the Indian estuaries have received wide attention in the recent past in order to understand the elemental composition of the sediments, the influence of anthropogenic activities on riverine chemistry and the transport of metals from rivers to the coastal oceans (Subramanianan 1980; Bijoy Nandan, and Abdul Azis, 1994; Satyanarayana et al 1993; Sesamal et al 1986l; Luoma et al 2008; Qasim and Sengupta (1981). The level of different metals is influenced by the type of effluents discharged by industries. Considerable enrichment of Hg was evident in the estuary of Mumbai when compared with the values reported for the open shore coastal water off Bassein–Mumbai (Zingde and Desai, 1981; Sahu and Bhosale, 1991). Similar increase has been reported from Ulhas estuary and the nearby Thane Creek due to the indiscriminate release of industrial effluents and domestic wastewater that goes largely untreated (Patel et al., 1985;; Zingde, 1999; Bhosale and Sahu, 1991). These toxic metals not only pollute the creek waters but also pose a threat to the aquatic biota. The heavy metal concentrations in Indian estuaries are much higher than the permissible limits ascribed

by international standards (Agadi et al., 1978; Nair S et al 1990; Alagarsamy, 2006; Ramkumar et al., 2012; Vardanyan and Ingole, 2006; Mohapatra and Rengarajan (2000). Food chain contamination by heavy metals has become a burning issue in recent years because of their accumulation in bio-systems from contaminated water, soil and air (Lokhande and Kelkar, 1999). The increase in residue levels of heavy metal content in water, sediments and biota, will result in decreased productivity (Lokhande and Kelkar, 1999). A comprehensive report on the metal pollution along the coastal and marine ecosystems of India has also sounded concern on deleterious effects of the ever increasing metal pollution on the marine biota (Pillai 1996). A comprehensive review of environmental geochemistry, mineralogical and mass transfer of metals of Indian river basins is available in literature (Subramanian, 1980; Subramanian et al., 1988 Sarin and Krishnaswami, 1984; Chakrapani and Subramanian, 1990; Sasamal et al 1987). Robin et al., (2012) observed that heavy metal concentrations in the marine environment of the Arabian Sea (except Cochin) were below the threshold levels associated with the toxicological effects and the regulatory limits. Metal enrichments were observed close to the major urban areas of coastal waters, mostly associated with large scale industrialization. In the Mandovi estuary particulate Fe (Kamat and Sankaranarayanan 1975) ; Al (Upadhyay and Sen Gupta 1995); As, Cu, Zn and Mn in marine flora and fauna of the estuarine waters (Zingde et al. 1976), trace metals (Alagarsamy et al, 2006) in the bottom sediments have been reported.

2.3.2 Metal –Microbe interaction

2.3.2.1 Ecological studies

The effects of heavy metals on macro-organisms are often apparent and well-documented but their effects on microbial communities have received less attention. Few studies based on culture method have been carried out from coastal waters, estuaries and deep sea. Tolerance to heavy metals was more pronounced in Gram-negative bacteria from coastal regions (Nair et al. 1993). The study showed that the marine chromogenic and non-chromogenic bacteria were resistant to heavy metals and drugs. Among the metals, Zn and Hg were found to be less toxic to pigmented bacteria than Cd. High frequency of metal resistance among pigmented bacteria may be due to the genetic linkage of these traits on the same plasmid or on co-transmissible plasmids. Pigmented bacteria were observed to be resistant only at higher

concentrations of antibiotics. Metal and antibiotics are linked to chromogenesis and differential sensitivity to both metals and antibiotics resistance were observed among pigmented and non-pigmented bacteria from the coastal waters (Nair et al.,1992). It was postulated that the pigmented bacteria would survive better in a metal-polluted environment, an analogue to the survival of Gram-negative bacteria (Olson and Thornton, 1982). Heavy metal tolerance in chromogenic and non-chromogenic marine bacteria was also recorded from the Arabian Gulf (Chandy, 1999). Hg tolerant bacteria are ubiquitous in the coastal waters of the Arabian Sea .and present even in the deeper depth (1000m) of the Bay of Bengal (De et al 2003, 2008). The population of Hg resistant bacteria along the Indian coast was 10^2 L^{-1} in water and 10^4 g^{-1} in sediment (Ramaiah and De, 2003). They were found in no-pollution (Positra, Marmogao, Terekhol, and Gopalpur), low-pollution (Malvan, Karwar, Paradip, Nagapattinam) and high-pollution (Mumbai, Chennai, Mangalore, Kulai, Padubidri, and Ratnagiri) regions (Ramaiah and De, 2003). They opined that the occurrence of Hg resistant bacteria in non polluted locations could be due to the adaptation of natural population. The gene responsible for metal resistant efflux pumps can be transferred from one bacterium to another through mobile genetic platforms such as plasmids and transposons (Ghosh et al., 2000; Gupta et al,1998, 2001). Cd showed negative effect on microbial biomass and diversity in a pond (Ganguly and Jana, 2002).They found that Cd affected the heterotrophic bacteria, ammonifying bacteria, ammonia oxidizing bacteria, denitrifying bacteria and cellulose decomposing bacteria. Very little information is available on the bacterial diversity from polluted Indian waters. Nithya et al., (2011) recorded metal resistant bacteria belonging to Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria (including alpha (α)-, gamma (γ)-, delta (δ)-, and epsilon (ε)-Proteobacteria), and uncultured bacteria in the Palk Bay sediments. Studies on bacterial tolerance levels of metals from different locations are available. Nair et al., (1993) studied the responses of two strains of bacterial isolates from Indian coastal waters and found that the growth of *Flavobacterium* sp. and *Bacillus* sp. were inhibited by Hg, Cd, and Zn..The order of inhibition observed for *Bacillus*sp was Hg>Zn>Cd and for *Flavobacterium* sp it was Hg>Cd>Zn.. Further, it was noticed that with prolonged incubation *Bacillus* sp. overcame the inhibitory effect whereas *Flavobacterium* sp. did not. Higher concentrations of glucose in the growth medium increased the inhibitory effect of the metal. At lower concentrations (15µg ml^{-1}) there was a stimulatory effect on [^{14}C] glucose uptake on *Flavobacterium* sp. Their study also showed that the Gram positive

isolate was less adaptable to metals than the Gram negative.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. The maximum level of tolerance exhibited by the bacterial isolates from the estuary was 1 mM in non-amended medium and 10 mM in 0.01% glucose amended medium (Krishnan et al., 2007) whereas bacteria from the coastal waters were capable of growth in liquid medium containing 50 ppm Hg (= 68 ppm $HgCl_2$) or more.Two isolates from an area with intense shipping traffic were found to be capable of colony formation on sea water nutrient agar with 75 ppm of Hg (Ramaiah and De, 2003). Mercury resistant marine bacteria could detoxify 70% Cd and 98% Pb from growth medium containing 100 ppm concentration of the metals (De et al. 2008). Apart from heavy metals, the mercury-resistant bacteria also degraded toxic polychlorinated biphenyls and tributylin (De and Ramaiah 2007). In the case of arsenite, estuarine bacterial strains could tolerate up to 1000 ppm tolerance and biotransformation potential showed the capability of detoxifying arsenic as much as 92% within 120 hr (Nagvenkar and Ramaiah, 2010). Studies from the deep sea regions were mainly on the tolerance level and the transformation of metals by bacteria (Sujith et al., 2010, 2014). The immobilization of Mn, Co and Ni by indigenous microbial communities associated with deep-sea sediments showed that suboxic and organically rich conditions favourMn and Co immobilization whereas oxic and organically poor conditions favour Ni immobilization. The general trend in immobilization of the metals after 45 days in organically richer core BC26 was Mn (85.6 μM g^{-1}) > Co (46.3 μM g^{-1}) > Ni (6 μM g^{-1}) n the other hand, the Mn(II) oxidizing bacteria associated with the basalt under ambient concentrations of organic carbon promoted the immobilization of Mn whereas carbon enriched conditions promoted the mobilization of Mn from the basalt surfaces. The mobilization of Mn in the presence of added glucose was 1.76μg g^{-1} d^{-1} (Sujith et al. 2014).

Two bacterial isolates CR35 and CR48 from the bottom waters of the Carlsberg Ridge had threshold concentration of 100 μM Mn^{2+} and precipitated Mnexternally (Fernandes et al., 2005). The maximum oxidation of Mn by CR35 isolate was 27 μM Mn d^{-1} in unamended medium and it was 35 μM Mn d^{-1} in 0.01% glucose amended medium by CR48 isolate. The same isolates when exposed to Ni and Co in the absence of Mn accumulated these metals both intra- and extracellularly (Sujith et al., 2010; Antony et al., 2011). Immobilization of Co by the bacterial isolates occurred at lower

concentration of 10 µM Co concentration (Antony et al., 2011) whereas Ni at 100 µM Ni^{2+} concentration stimulated the growth of the bacteria. The immobilization of Ni by CR35 isolate was 1.49 µM d^{-1} and for CR48 isolate it was 0.12 µM d^{-1} (Sujith et al., 2010). The immobilization of Ni showed the participation of hydroxyl, carbonyl, sulphide and phosphoryl groups in Ni binding. With Co, change in morphology of cells was observed, a strategy to counter the toxicity of the metal. Isolates from the Afanasiy-Nikitin Seamount tolerated higher concentration of Co, but showed decreased level of tolerance in the presence of glucose (Sujith et al., 2010). In India, industrial developments have resulted in severe disturbance of ecological balance in most ecosystems. The outcome of the disturbances include a decreased biodiversity, an irreversible extinction of many sensitive species and the selection of cosmopolitan ones that are adapted better to contemporary environment.

2.3.2 Cochin Estuary

. The Cochin backwater (Cochin estuary) is the largest of its kind on the west coast of India with an area of 256 Km^2. It is a well-studied estuary and its hydrography, chemistry and biology was reviewed by Qasim(2003). The hydrography, flow regime and geochemistry of CE have been well described by several researchers (Sankaranarayananet al., 1998; 1978; Paul and Pillai., 1983;, Nair et al, 1990; Ouseph, 1992). Trace metals in bulk water and sediment were analyzed recently and the concentration showed an increase from the earlier reports (Manju et al., 2014, Deepulal et al., 2012, Martin et al., 2012, Priju and Narayana 2007, Kumar et al., 2010). The concentration of trace metals in the sediments of Vembanad estuary, Muvattupuzha river (Mallik 1987; Padmalal ,1997) and also the surface sediment of CE have been investigated (Paul sk 2001; Balachandranet al.,2005 and 2006; Renjith and Chandramohanakumar, 2007). Apart from the natural processes, the Cochin estuary receives an additional dosage of effluents through industrial and domestic discharges (Balachandran et al., 2006; Nair et al., 2006). There are 16 major and several minor industries situated in the upstream region of the Cochin estuary discharging nearly 0.105 Mm^3d^{-1} of effluents. The effluents contain fertilizer, pesticide, radioactive mineral, chemicals, petroleum products, heavy metal and fish processing discharges. Apart from the natural processes, the Cochin estuary receives

an additional dosage of heavy metals through industrial and domestic discharges. (Balachandran et al., 2006; Nair et al., 2006). Several monitoring programs have been initiated to understand the level of heavy metal pollution and its bioaccumulation which have significant effect on the health of the estuaries, its inhabitants and the coastal waters. Studies in the Cochin estuary during the past three decade have demonstrated an increase in Zn concentration in the sediments from 70 to 1266 mg kg^{-1} (Venugopal et al 1982; Balachandran et al 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). This significant increase in the heavy metal concentration (Balachandran et al., 2005; Nair et al., 2006; Paul sk, 2001) is mostly through increased discharge of industrial and domestic wastes (Balachandran et al., 2006; Nair et al., 2006). A study on the distribution of metals in the sediment core in the Cochin estuary showed an increase in their concentration from bottom to the surface, which further confirms the increased anthropogenic activities with time (Unnikrishnan and Nair, 2004). High level of heavy metal have been reported in bivalve (Lakshmanan et al 1989, Pillai et al 1995, Nair and Nair 1986), benthos (Jayaraj, 2006) and fish (Nair et al., 2006) from the Cochin estuary. Although studies on microbial ecology have been initiated in CE (Thottathil et al 2008a,b; Parvathi et al., 2009; Parvathi et al., 2011; Thomas et al., 2006;Chandran and Hatha, 2003)studies on metal - microbe interaction have hitherto not undertaken. The effect of heavy metal pollution on the diversity and dynamics of microorganisms in the marine environment is a topic of growing environmental concern as it can provoke unintended alterations in the functioning of this estuary directly or indirectly.

Chapter 3
MATERIALS AND METHODS

Contents

3.1. Study Area

The Cochin estuary (CE) is one of the largest and productive estuaries along the south west coast of India.The average depth is 5±2 m, except in the mouth region of the estuary where the depth varies from 10 to 13 m. It serves as a repository for effluents discharged from various industries such as fertilizer, pesticide, radioactive mineral and heavy metal processing units , chemical and allied industries, petroleum refineries and fish processing units (Report submitted by Monitoring Committee on Hazardous Wastes and Hazardous Chemicals set up by the Supreme Court. http://www.pucl.org/ Topics/Industries-envirn-re-settlement/ 2004/ eloor.htm). These effluents are rich in heavy metal concentrations such as zinc, cadmium, nickel, cobalt and copper.

3.1.1. Sampling locations

Three sampling locations, viz.Vypin, Munambam and Eloor were selected for the study based on the concentration of heavy metals reported at these locations (Balachandran et al., 2005). The geographical positions of the sampling sites are shown in Figure 1. Eloor is at an intersection where the river Periyar joins the CE. It receives substantial input of untreated effluents from more than 240 industries located along the banks of the river Periyar. Vypin is situated close to the mouth of the estuary which is the major opening through which the Arabian Sea water enters into the estuary. Average depth of Vypin is 10 m and is relatively not influenced by industrial inputs. Munambam is located at the northern end of the estuary and has a fishing harbor. The average depth is 4 m and is categorized as a saline area.

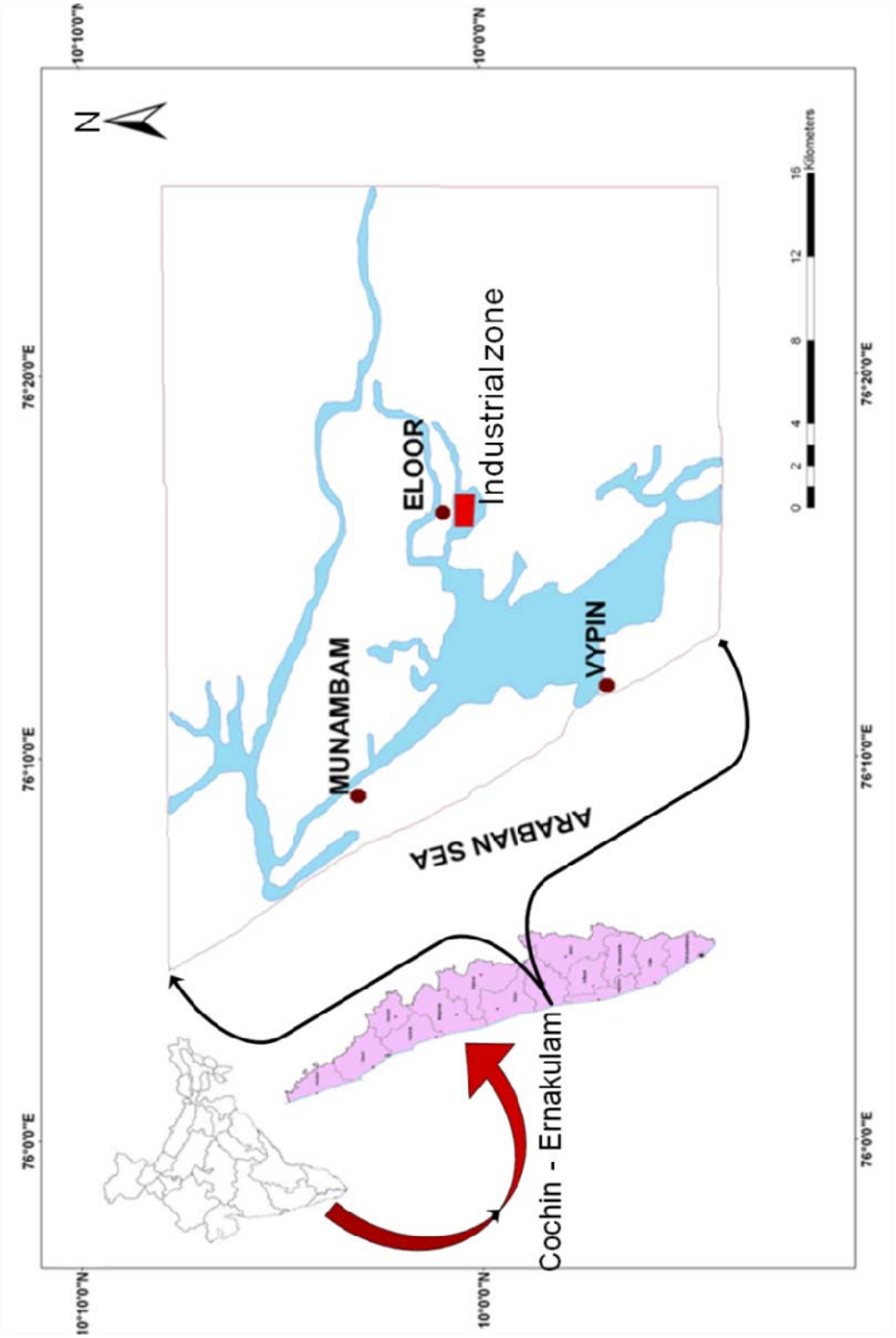


Figure 1 Map showing station locations in CE

3.1.2 Sample collection

Sediment and water samples were collected in March 2008 from the three locations. From each location, five sediment samples were collected using a van Veen grab (Mouth area - 0.05 m^2). Sub-samples of the sediments were collected in plastic bag using alcohol sterilized plastic spatula to avoid metal contamination. Near-bottom watersamples were collected from the 3 sites by using a 10 L capacity Niskin water sampler. The sampler was closed with a messenger at the desired depth. For metal analysis all precautions were taken to avoid contamination from all possible sources. Nylon rope was used for operating the samplers. Samples were collected in pre-cleaned and acid washed polyethylene containers. All the samples were transported in an ice chest to the laboratory for analysis.

3.2 Analysis of Environmental Parameters

3.2.1 Water

The water samples from the 3 locations were analyzed for routine environmental parameters. Standard curves for each analysis are in the Appendix section.

3.2.1.1. Salinity and Temperature

Salinity and temperature were measured onboard with the aid of a portable Conductivity-Temperature-Depth (CTD) probe (Sea Bird Electronics, Inc., USA).

3.2.1.2. Dissolved oxygen (DO)

Dissolved oxygen was determined by the Winkler's method by Strickland and Parsons, (1972) using standard iodimetric titration. The principle of the determination and the possible sources of systematic errors are discussed by Grasshoff,(1983). Water samples were collected from the Niskin sampler in 125 ml acid washed (10% HCl) glass-stoppered bottles taking care that no air bubbles were trapped inside and fixed immediately on board with 1 ml of manganous chloride and 1 ml of alkaline iodide solution (Winkler's reagents A & B respectively). The samples were mixed thoroughly and the precipitate was allowed to settle. The samples were transported to the laboratory for analysis. One ml of sulphuric acid (10 N) was added to each sample and the sample was shaken thoroughly for dissolving the precipitate and was titrated against 0.01 N

sodium thiosulphate using starch as the indicator. The procedure was standardized by using potassium iodate.

3.2.1.3. Nitrite

In this method, nitrite in the water sample when treated with sulphanilamide in acid solution(Appendix) results in a diazo compound, which reacts with n(l-naphthylethylenediaminedihydrochloride to form an azo dye (Grasshoff, 1983). The absorbance was measured at 543 nm.Standards were run with analytical reagent quality sodium nitrite. (precision: (0.01 µmol $N\text{-}NO_2^-l^{-1}$).

3.2.1.4. Nitrate

Nitrate-N in the water sample was quantitatively reduced to nitrite by passing through a reduction column filled with copper coated cadmium granules and measured as nitrite. During the reduction stage, ammonium chloride buffer was added to the sample to maintain a stable pH (Grasshoff, 1983). The sample after reduction was analyzed for nitrite-N as described in section 3.2.1.3. (0.1 µmol $N\text{-}NO_3^-l^{-1}$).

3.2.1.5. Phosphate

Phosphate-P was determined as inorganic phosphate by the formation of a reduced phosphomolybdenum blue complex in an acid solution containing molybdic acid, ascorbic acid and trivalent antimony. The method was developed by Murphy and Riley, (1962) and a variation of this method described by Grasshoff, (1983) was adopted in the present work. Instead of single solution reagent as in the Murphy and Riley procedure, two stable reagent solutions were used here. A mixed reagent of 0.5 ml containing molybdic acid and antimony tartrate followed by 0.5 ml of ascorbic acid reagent were added to 25 ml aliquots of the samples. The absorbance was measured at 882 nm within 30 minutes to reduce any possible interference from arsenite.Potassium dihydrogen orthophosphate was used as standard.

3.2.2 Metal Analysis

Concentrations of Zn, Hg, Co, Cd, Cuand Ni in the water and sediment samples were analyzed using inductively coupled plasma atomic emission spectrometer (ICP-AES - Thermo Electron IRIS INTREPID II XSP DUO). Sediment samples (5 nos.) from each

station were sub-sampled and pooled for analysis. The pooled samples were dried at 60 ^{0}C and finely powdered using mortar and pestle. One gram of finely powdered dried sediment was digested repeatedly with HF-$HClO_4$- HNO_3, suspended in 0.5 M HCl (25 ml) and analyzed for heavy metals following the standard protocol (Loring and Rantala, 1977). For water samples, a known volume was filtered through pre–weighed Millipore filter paper (0.45 µm) and the filtrate was acidified using concentrated HCl. The dissolved metals were extracted using 2 % ammonium pyrrollidinedithiocarbamate (APDC) in 10 ml of methyl Isobutyl ketone (MIBK) at pH 4.5 and brought back to aqueous layer by back-extraction with concentrated HNO_3 and made upto 20 ml with sterile de-ionized water (Smith and Windom, 1972). The extracts were analyzed in the flame for trace metals. The analyses were done in triplicate. The concentration of metal in water and sediment are expressed as mg L^{-1} and mg kg^{-1}, respectively.

3.3 Bacterial Variables

3.3.1 Abundance

Four fractions of the bacteria viz. total counts, total direct viable, total culturable counts (colony forming units) and metal resistant bacterial counts were enumerated. The details of the media composition and reagents used are given in the Appendix section.

3.3.1.1 Total Counts (TC)

Water samples were fixed in hexamine buffered formalin (2%) and diluted ten times in sterile saline. For sediment samples, one gram of the pooled sediment sample in hexamine buffered formalin (2%) was dispersed in sterile saline and sonicated in a water bath for 10 min to dislodge the cells. Enumeration was done following the method of Hobbie et al., (1977). Preserved samples (1 ml) were stained (in duplicate) with 100 µl of acridine orange stain (Hi-Media, Mumbai) (final concentration 0.01% w/v) and incubated in dark for 2 minutes before filtering through 0.22 µm black stained Nuclepore polycarbonate membrane filter (Whatman Asia Pacific, Singapore). The slide was then viewed under oil immersion objective (100x) of an epifluorescence microscope (Olympus Corporation, Japan), equipped with HbO lamp and U-MWB2 mirror unit having excitation filter of 460–490 nm and emission filter of 520 nm. About 10-12 fields of >30 bacteria per field were counted per filter using 100X oil

objective lens and the average field count was used to calculate the total bacterial abundance using the equation given below. The bacterial abundance in sediment and water are expressed as cells g^{-1} or cells $L^{-1,}$ respectively.

$$\frac{\text{Filter area}}{\text{Area of counted field}} \text{ X Average count X } \frac{1}{\text{Volume of sample}} \text{ X } \frac{1}{\text{Dilution}}$$

3.3.1.2 Total Viable Count (TVC)

Total viable count was enumerated following the method of Kogure et al., (1987). The samples were incubated under dark at 28 ± 2°C for 8 hours (hr) with pre-sterilized yeast extract solution to a final concentration of 0.01 % and 50 µl of antibiotic cocktail. The samples were fixed in hexamine buffered formalin (2%). Counting procedure was the same as mentioned in section 3.3.1.1 Swollen and elongated cells were enumerated as viable bacterial cells and expressed as cells g^{-1} and cells L^{-1}, respectively for sediment and water.

3.3.1.3 Culturable Aerobic Bacteria

Culturable bacterial abundance was enumerated on Nutrient agar (NA) and Peptone Yeast extract Tryptone (PYT80) agar plates (Appendix). The samples were serially diluted and 500 µl of the samples were spread plated on NA and PYT 80 agar plates. Colony forming units were counted after incubating the plates at 28 ± 1°C for 48 hr. The numbers are expressed as cfu L^{-1} for water and cfu g^{-1} for sediment.

3.3.1.4 Metal Resistant Bacteria (MRB)

The metal resistant bacteria were enumerated using the method of Hassen et al., (1998). Briefly, PYT 80 agar plates supplemented with 0.005 mM concentration of each metal ($ZnSO_4.7H_2O$, $HgCl_2$, $CoCl_2.6H_2O$, $CdCl_2$, $CuSO_4.5H_2O$ and $NiCl_2$) were prepared. The samples after serial dilution were spread plated and incubated at 28 ± 2°C for 7 days. The colonies appeared on these plates were considered as MRB. The colonies were counted and expressed as cfu L^{-1} for water and cfu g^{-1} for sediment.

3.4. Studies on MRB

Morphologically different metal resistant bacteria (250 nos) were isolated from different metal plates and purified on NA plate following streak plate method. The

purity of isolates was confirmed by Gram staining. The purified isolates (Vypin 83 nos., Munambam 83 nos. and Eloor 84 nos.) were stored in NA slants amended with metal (0.005mM) at 4°C for further analysis.

3.4.1 Identification

MRB were identified based on the fatty acid profile of the bacterium developed for aerobic bacteria by MIDI Inc. USA)(Sasser, 1990). The details of reagents used for fatty acid extraction are given in the Appendix section. Briefly, the bacterial isolates were grown on Trypticase soya (TS) agar plates for 24 hr at room temperature (28 ± 2°C). Wet bacterial biomass of 40 mg was saponified at 95-100°C for 5 min in 1 ml saponification reagent. After 5 min., the reaction mixture was vortexed and continued the saponification for 25 min. Subsequently, the tube was supplemented with 2 ml methylation reagent and kept at 80 ± 1°C in water bath for 10 min. The mixture was extracted with methanol: methyl tert-butyl ether (1:1) in a laboratory rotator and the aqueous phase was discarded. The remaining fraction was subjected for a base wash with NaOH (0.25 M) in distilled water (900 ml) and the extract was transferred in to a GC sample vial. Fatty acid was analyzed in Agilent GC 6950 by injecting 2 µl of sample through a 25 m silica capillary. The oven temperature was increased from 170°C to 310°C during each run. By increasing the temperature, the fatty acids get volatilized at specific temperature and detected in a Flame Ionization Detector (FID). CHEMSTATION software converted the signal from FID into a chromatogram, which was transferred to SHERLOCK software for comparing with fatty acid profile library of known organisms. SHERLOCK software has an inbuilt library of fatty acid profiles for around 3000 bacterial species (Pendergrass and Jensen, 1997).The organisms were identified based on the similarity index. The chromatogram and composition report of a representative bacteriium is given in Appendix . Previous studies have shown that more than 90 % identification of bacteria by fatty acid profile is in accordance with 16S rRNA gene sequencing method at the genus level and more than 70 % at the species level (Osterhout et al., 1991; Tang et al., 1998).

3.4.2 Multiple Metal Resistances

Bacterial resistance to heavy metals was examined by the plate diffusion method (Hassen et al., 1998). The glassware used in the experiments were leached in 2 N HNO_3

and rinsed several times with sterile de-ionized water before using to avoid metal contamination. In the central well of PYT plates, 500 µl of 5 mM of metal solution ($ZnSO_4.7H_2O$, $HgCl_2$, $CoCl_2.6H_2O$, $CdCl_2$, $CuSO_4.5H_2O$, $NiCl_2$) was added and incubated at 28±2°C for 24 hr to allow diffusion of the metal into the agar. Six strains of bacteria were streaked in a radial fashion on each metal plate (in triplicates) and incubated for 7 days at 28 ± 2°C. Bacteria which showed visible growth were counted as metal resistant and scored for multiple resistances.Microorganisms were also screened for $AgNO_3$ resistance in LauriaBertani (LB) agar plates supplemented with different concentrations of $AgNO_3$, ranging from 0.5 mM to 1000 mM. Filter sterilized $AgNO_3$ solution was added to the LB agar medium just before preparing plates. Spot inoculation of 10 bacteria were done on a single plate and incubated in the dark at room temperature. The isolates showing growth in 72 hr was considered as resistant strains.

3.4.3 Antibiotic Resistances

Sensitivity of MRB to 12 commercially available antibiotic discs (Hi- media, Mumbai) was tested. The mode of action, chemical class and concentration of antibiotics are given in Table 1.

Mode of action	Chemical Class	Name	Abbreviation	Concentration per Disc (µg)
Cellwall synthesis	β-Lactams	Ampicillin	A	25
	Glycopeptides	Vancomycin	Va	10
Protein synthesis	Macrolides	Azithromycin	At	15
	Tetracyclines	Oxytetracyclin	O	30
		Tetracyclin	T	10
	Aminoglycosides	Gentamycin	G	30
		Amikacin	Ak	10
	Chloramphenicol	Chloramphinicol	C	30
Nucleic acid synthesis	Quinolones/ Fluoroquinolones	Ciprofloxacin	Cf	10
		Nalidixic acid	Na	30
	Nitrofurantoin	Nitrofurantion	Nf	100
	Sulfonamides	Trimethoprim	Tr	10

Table 1 Details of the antibiotics used in the study

Muller Hinton agar plates were swabbed with 0.5 ml overnight grown cultures and the antibiotic discs were placed aseptically. A maximum of six discs were placed at sufficient distance from each other and all cultures were tested in duplicate. The plates were incubated at 28±1°C for 18 hr and the clearing zones formed around the discs were recorded using Hi Antibiotic Zone Scale (Hi media,Mumbai). The isolates were recorded as resistant if there was no visible zone around the discs. Multiple Antibiotic Resistance (MAR) index was calculated as the number of antibiotics to which each isolate was resistant to the total number of antibiotics tested (Krumperman and Paul1983).

3.4.4 Enzyme Expression

The presence ofenzyme among MRB was tested in NA medium prepared with 50% sea water supplemented with different substrates. MRB were grown in NA overnight and the active cultures were used for different enzyme assay. Presence of enzyme was scored based on the clearing zone or change in colour. The extent of enzyme expression or production was measured as the function of zone size of individual MRB to produce clearing zone in the substrate enriched solid media. Based on the zone size (diameter in mm), production by MRB was classified as Low (0-10 mm), Medium (11-20 mm) and High (> 20 mm).

3.4.4.1Amylase

The bacteria were spot inoculated over the surface of a NA plate supplemented with 0.2 % soluble starch and incubated for 24 hr at 28 ± 2°C. The ability of bacteria to produce amylase was identified by flooding the plates with 1% iodine solution. The zones of clearance around the colonies were measured.

3.4.4.2 Gelatinase

The colonies were spot inoculated over the surface of a NA plate supplemented with 0.4 % gelatin and incubated at 28 ± 2°C for 48 hr. The ability of bacteria to utilize animal protein gelatin was recorded as the clearing zone around the colonies on addition of $HgCl_2$ solution (15%).

3.4.4.3 Lipase

NA medium was supplemented with 0.01% $CaCl_2$. Tween 80 was sterilized by autoclaving at 121°C for 20 min and was added to the molten agar medium at 45-50°C to give a final concentration of 1% (vol/vol). The medium was shaken well until the Tween was thoroughly mixed and was dispensed into petri plates and solidified. The cultures were spot inoculated and incubated at 28 ± 2°C. After 72 hr, the plates were observed for formation of an opaque zone containing crystals of calcium around the colonies as positive for lipase activity.

3.4.4.4 DNase

The ability of bacteria to produce DNase was tested using readymade DNase agar (Hi media, Mumbai). The composition of the medium is given in Appendix section. The cultures were spot inoculated and incubated at 28 ± 2°C for 24 hr. The formation of blue coloured colonies due to toludine blue was considered as postive for DNAse.

3.4.4.5 Phosphatase

Phenolphthalein phosphate agar containing sodium phenolphthalein phosphate (0.01%) was prepared in 50 % sea water. The cultures were spot inoculated and incubated at 28 ± 2°C. After 48 hr of incubation, the colonies were subjected to ammonia fumes and the formation of pink color to the colonies in 3 seconds (sec) was considered as positive.

3.4.4.6 Urease

Christensen's urease agar medium was prepared in 50 % sea water and sterilized at 115°C for 20 min. Filter sterilized urea (40%) was added to the sterilized medium and mixed well before pouring to plate. The plates were spot inoculated and incubated for 48hr at 28 ± 2°C. Development of pink color around the colony was considered as positive.

3.4.4.7 Protease

Skim milk (5%, final concentration) incorporated in nutrient agar were used as the screening medium. The cultures were spot inoculated andincubated at 28 ± 2°C. Clear zone around the bacterial colony afterincubation indicated hydrolysis of casein.

3.5. Effect of Silver Nanoparticles (AgNPs) On Mutiple Drug Resistant (MDR) Pathogenic MRB

3.5.1 Selection of MDR pathogenic MRB

Five MDR pathogenic MRB were selected for this study. Out of which three were Gram negative (*Vibrio alginolyticus, Pseudomonas aeruginosa* and *Escherichia coli*), and two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilus*). These bacteria showed high levels of resistance against heavy metals and antibiotics.

3.5.2 Preparation of AgNPs

AgNPs was provided by Prof. Anantharaman, Department of Physics, Cochin University of Science and Technology, Kochi, Kerala. The preparation and characterization of the nanoparticles were done based on Thomas et al., (2008). Briefly, AgNPs was prepared using Ag–SiO_2 solution by employing tetraethyl orthosilicate-(TEOS), ethanol, distilled water and silver nitrate as precursors. The mean particle size was 5–6 nm. The concentration of silver present in different volumes used in this study was 0.189 ppm in 20µg.ml^{-1}, 0.208 ppm in 40µg.ml^{-1}0, 153 ppm in 60µg.ml^{-1}, 0.136 ppm in 80µg.ml^{-1} and 0.126 ppm in 100µg.ml^{-1}.

3.5.3 Effect of AgNPs onMDR pathogenic MRB

To study the effects of nanoparticles on MDR, pathogenic MRB,log phase cells of the cultures were treated with AgNPs (in triplicate) for 3 hr. The controls maintained were cultures without AgNPs. Both the experiment and control MDR pathogens were analysed for cell wall integrity, viability, metabolically active cells, fatty acid composition of the whole cell and genetic stability.

3.5.3.1 Cell wall integrity

Scanning Electron Microscopic analysis

Control and treated cell pellets were subjected to dehydration by running it through a series of increasing concentrations of acetone (10, 30, 50, 70, 90 and 100%). The samples were air dried, mounted on a stub and sputter coated with Au/Pd. The cell wall integrity was observed using Scanning Electron Microscope (SEM).

3.5.3.2. Viability

The loss of viability of bacteria (death) was tested using 2 methods viz. 1) Resistant population (%) and 2) SDS assay.

1. Resistant population

Overnight grown cultures in LB broth were dispensed separately into microcentrifuge tubes to obtain a final concentration of 10^6cells ml^{-1}. The tubes were exposed to 0, 20, 40, 60, 80 and 100 $\mu g.ml^{-1}$ concentrations of AgNPs separately for one hour, and were spread over the surface of LB agar plates. All plates were incubated at 28±1 ^{O}C for 24 hr before enumerating the resistant population (%).

2. SDS Assay

Overnight cultures of bacterial cells were washed copiously with sterilized phosphate buffered saline (pH 7.4) and 200 µl of the washed cells were dispensed in the wells of sterile micro plate. After measuring the initial absorbance, the test solution was supplemented with, AgNPs (100 μgml^{-1}) and SDS (0.1%). Control wells without AgNPs and SDS were also maintained. The absorbance at 600 nm was recorded every 15 min for a period of 2 hr. The percentage decrease in absorbance compared to the initial reading was plotted against time (Lok et al., 2006)

3.5.3.3 Metabolic active cells

Bacterial cells were treated with AgNPs for one hour and stained with 20 µl of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) solution following the procedure recommended by the manufacturer (Molecular Probes; Invitrogen, Eugene, OR, USA). Experimental and control samples were incubated for 30 min in dark condition. Samples were filtered through 0.2 µm polycarbonate filters, and counter stained with 4',6-diamidino-2-phenylindole (DAPI). Counterstaining with DAPI allowed concurrent determination of total (i.e., viable plus non-viable) and respiring (i.e., cells exhibiting CTC-formazan fluorescence) cell counts in a single preparation. The samples were washed copiously with phosphate buffered saline and the dead and metabolically active cells were differentially counted using 100 X oil objectives under an epifluoresence microscope (Olympus, USA). Metabolic active cells were

expressed as the ratio of metabolically active cells (i.e., CTC stained) to total cells (i.e., DAPI stained, cells). If all the cells were metabolically active, then the ratio of dead to live cell would be close to one.

3.5.3.4 Whole ell fatty acid composition

The whole cell fatty acid profiles of pathogenic bacteria before and after treatment with AgNPs were analyzed using a gas chromatography system (16890N Network GC system, Agilent Technologies Inc., Wilmington, DE). Difference in the number and the intensity of fatty acids were compared. The details of fatty acid analysis are as described in section 3.4.1.

3.5.3.5 Genetic stability

The effect of AgNPs on stability of genetic material of pathogens was investigated by comet assay (Singh et al., 1999).Details of reagent preparation is given in Appendix. Briefly, AgNPs treated and control cells were washed copiously with phosphate buffered saline (PBS, pH 7.4) and re-suspended in saline and subjected to comet assay. For comet assay, 50 µl of 10^6 bacterial cells were mixed with 500 µl of 0.5% low melting point agarose prepared in Tris acetic acid EDTA buffer (TAE). Bacterial cells impregnated in agarose solution were spread over a microscopic slide, pre-coated with a thin layer of 0.5% agarose and solidified by incubating at 4^0C for 30 mins. Slides were then incubated at 37^0C for 30 min and subjected to cell lysis for 1 hr at 37^0C, by immersing in lysis solution. Subsequently, the cells were subjected for enzyme digestion by incubating in enzyme solution for 2 hr at 37^0C. After digesting the cell wall by lysis and enzyme treatment, the slides were equilibrated with 300 mM sodium acetate and subjected to electrophoresis at 50 V for 15-20 min. Following electrophoresis, slides were immersed in 1 M ammonium acetate in ethanol for 30 min and then in absolute ethanol for 1 hr. The slides were air dried at room temperature (28±1ºC) and then immersed in 70% ethanol for 30 min and air dried. Slides were then stained with 1 ml freshly prepared solution of 1 µl SyBr green in 1ml Tris EDTA (TE) buffer. The comets were recorded using a fluorescence microscope (OLYMPUS BX 61) equipped with dichroic filter pairs (Excitation filter: 470-490 nm, Emission filter 520 nm, Dichroic 500 nm) and digital camera attached to fluorescence

microscope. The comet length was measured and processed using the software Image Pro. The nucleic acids were classified based on comet length as intact/low (0 -10 mm length) or heavy damaged (>10 mm) groups and are presented as histogram.

3.5.4 Mechanism of resistance by MDR *Staphylococcus aureus*

To understand the resistance mechanism of MDR strain *S. aureus* to AgNPs resistance, Verapamil assay was carried out. Verapamil is a known calcium channel blocker for ABC transporter. *S. aureus* cells were exposed to Verapamil to a final concentration of 20 μg ml^{-1} for 1 hr prior to the treatment with AgNPs. Total death rate and nucleic acid damage of the treated cells were determined following the method given under sections 3.5.3.2. and 3.5.3.5 respectively.

3.6 Statistical Analysis

Metal concentrations of water and sediment, abundance, community structure of culturable and non-culturable bacteria were subjected to various statistical analysis (Snedecor and Cochran, 1967). Depending on sample size and distribution, the data were normalized. The analyses were carried out using, student's t test, 3 ways ANOVA. Diversity index (Shannon-Wiener H') for culturable community (Shannon and Weaver, 1963) and Cluster Analysis were done using Primer 6 software.

Chapter 4
RESULTS

Contents

4.1 Water quality

The physico-chemical characteristics of the near-bottom water from the 3 locations are given in Table 1. There was not much variation in the overall characteristics of the water at the 3 stations and is comparable to earlier studies during the same period in CE. Eloor is in the upper region of the estuary and has direct riverine influence unlike Vypin, which is at the mouth of the estuary. Eloor recorded low salinity (19) and high nitrate (19.35 µmol N-$NO_3^{-}l^{-1}$) compared to Vypin and Munambam.

Sl No	Parameters	Vypin	Munambam	Eloor
1	Temperature (^{0}C)	29	31	30
2	Salinity	31	27	19
3	Dissolved Oxygen (ml l^{-})	3.16	3.9	5.17
4	Nitrite(µmolN-$NO_2^{-}l^{-1}$)	0.73	0.37	0. 20
5	Nitrate(µmol N-$NO_3^{-}l^{-1}$)	3.82	10.70	19.35
6	Phosphate (µmol P-$PO_4^{3-}l^{-1}$)	1.38	0.33	0.51

Table 1 Physico-chemical characteristics of near-bottom water at the 3 stations

Variation in metal concentrations

The average concentrations of heavy metals in water and sediment at Vypin, Munambam and Eloor are presented in Table 2. Out of the six metals analysed, except Hg other five metals were detected in the sediment and water. In sediment, concentration of Zn was maximum (mean = 1017.7 mg kg^{-1}) followed by Ni (mean = 122.67 mg kg^{-1}). Among the metals Zn was the most variable (C.V %= 121.5) and

cobalt was the least variable (C.V % = 58.04).The concentration of all metals was less in water compared to the sediment but the pattern of distribution of metals was similar in water and sediment. In water Zn was the major metal (413.6 $\pm$ 528.04mgL^{-1}) followed by Ni and Co. Ni showed least variability (c.v %=83.14) whereas the other metals are distributed with high variability (C.V % 127.7 - 141.4). Among the three locations, Eloor recorded higher concentrations of all metals both in the sediment and water. The concentration of Zn at Eloor was as high as 2758 mg kg^{-1} and 1159 mg L^{-1} in sediment and water, respectively. At Vypin, only Co (16 mg kg^{-1}) was detected in the sediment, whereas rest of the metals were below the detection limit in both water and sediment. There was a gradient not only in the concentration of metals but also in the number of metals recorded at the 3 stations.

Heavy Metals	Sediment (mg kg^{-1})			Water (mg L^{-1})		
	Vypin	Munambam	Eloor	Vypin	Munambam	Eloor
Zn	BDL	290	2758	BDL	80	1159
Co	16	80	114	BDL	21	50
Ni	BDL	109	259	BDL	56	123
Cu	BDL	42	145	BDL	BDL	97
Cd	BDL	27	164	BDL	BDL	50
Hg	BDL	BDL	BDL	BDL	BDL	BDL

Table 2. Heavy metal concentration in sediment and water at the 3 stations (BDL-below detection limit)

3 way ANOVA of metal concentrations showed significant difference between water and sediment ($F_{(1,10)} = 5.144$ $P<0.05$), between metals ($F_{(6,10)} = 7.784$, $P<0.05$) and between stations ($F_{(2,10)} = 10.01$ $P<0.05$). Metal-station interaction was also high ($F(10,10)=6.068$, P,0.05) showing spatial selectivity for metals except Zn.

Cluster analysis (Figure 1), grouped the stations according to the metal concentrations and the clusters were statistically different. Sediment sample from Eloor was distantly separated from other 2 stations. Water sample from Eloor and sediment sample from Munambam were in the second cluster. This clearly shows that the metals were carried down the estuary from source point (head of the estuary). The metal concentrations in the 3 stations corroborates with the earlier report that classified Eloor as heavily, Munambam as moderately and Vypin as least polluted stations in the CE.

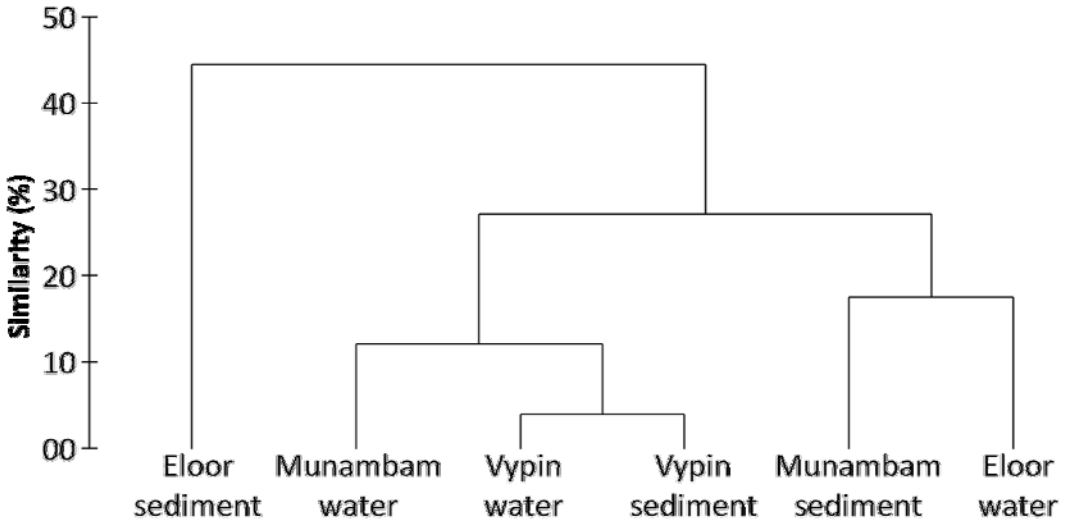


Figure 1. Dendrogram of the stations Heavy metal concentrations were used to link the stations

4.2 Bacterial Abundance

4.2.1 TC, TVC and TCC

The bacterial abundance at the three stations is presented in Figure 2. TC in sediment ranged from 10^9 to 10^{10} cells $g^{-1}$and in water from 10^8 to 10^9 cells L^{-1} at the 3 stations. TC was $2.23 \pm 0.3 \times 10^9$ and $6 \pm 0.05 \times 10^{10}$ cells g^{-1}in the sediment of Eloor and Vypin respectively whereas it was $1.15 \pm 0.1 \times 10^9$ and $6.5 \pm 0.05 \times 10^8$ cells L^{-1} in the water, respectively. In Munambam, TC in the sediment was$1.09 \pm 0.3 \times 10^9$ cells g^{-1}. TVC was four order less than TC and ranged from 10^4 to 10^5 cells L^{-1} in water and 10^5 to 10^6 cells g^{-1} in sediment. TVC in the sediment was high at Vypin and Munambam. It ranged from $1.05 \pm 0.4 \times 10^5$ to $2.65 \pm 0.3 \times 10^6$ cells g^{-1}. TCC, was one to two order less than that of TVC. Though Eloor recorded comparative less bacterial population, there was no significant variation in TC, TVC and TCC between stations in water ($P > 0.05$) and sediment ($P > 0.05$).

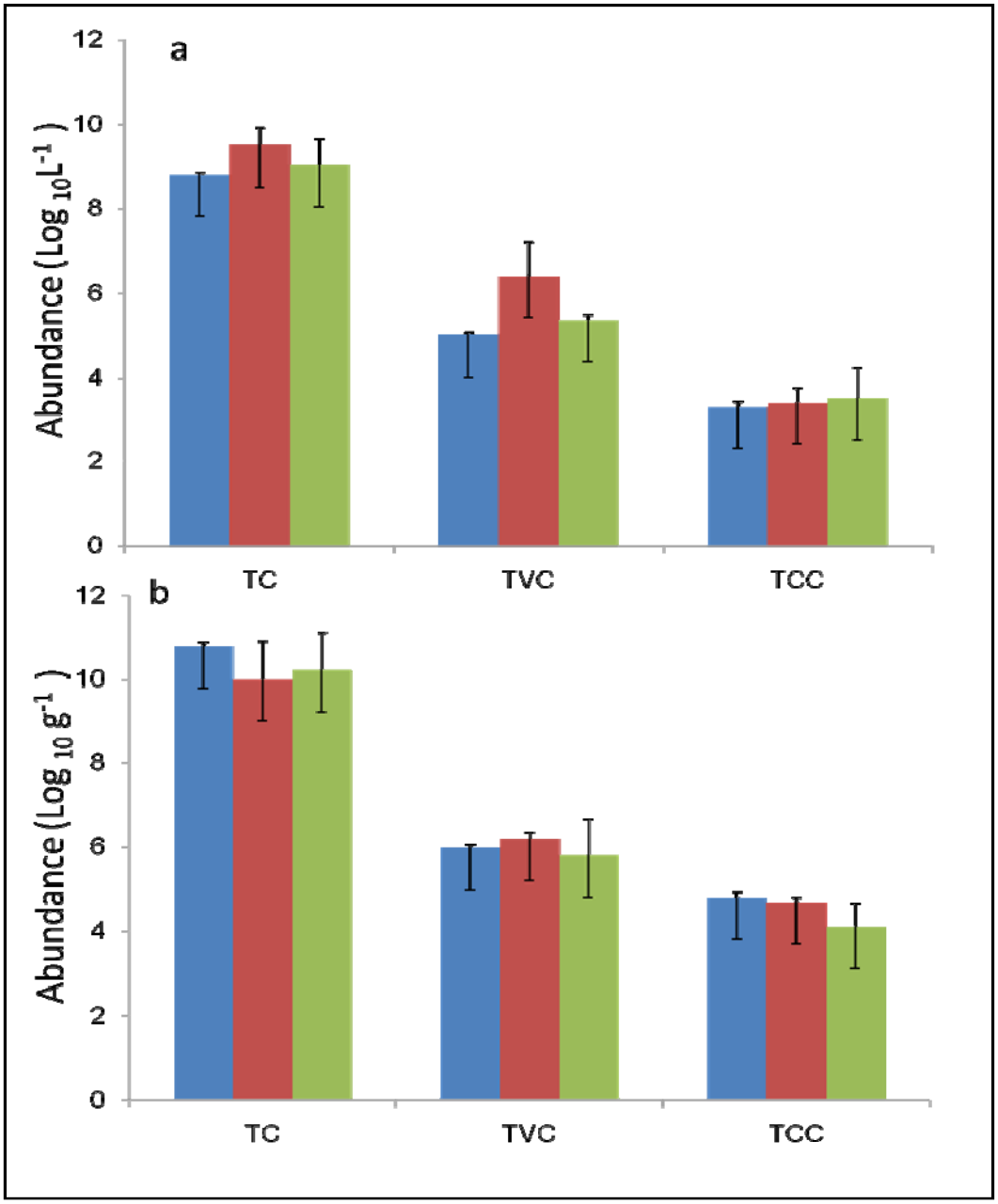


Figure 2. Bacterial abundance at Vypin( )Munambam( ) and Eloor( ) stations (a) water and (b) sediment Values are expressed as average ± SD

4.3 Studies on MRB

4.3.1 Abundance

MRB population in different metal amended media ranged between 10^{2-4}cfu g^{1} in sediment and 10^{2-3}cfu L^{-1} in water (Table 3,4). At Eloor MRB population was high in the sediment and ranged between 2.5 ± 0.13 x 10^{4} to 6.3 ± 0.4 x 10^{2} cfu g^{-1}. Though Hg was below detection limit in sediment and water Hg -MRB abundance

was 6.3 ± 0.4 x10^3cfu g^{-1} in sediment and 4.2 ± 0.9 x 10^2 cfu L^{-1} in water at Eloor (Table 3,4). At Vypin and Munambam the MRB was one order less than Eloor, and was in the range of 10^3cfu g^{-1} and 10^2 cfu L^{-1} in sediment and water, respectively. There was significant difference between stations in the MRB population of water ($P< 0.05$) and sediment ($P < 0.0005$). There was also significant difference in MRB population between different metal in water ($P< 0.005$) and sediment ($P < 0.015$). The abundance of MRB appears to be influenced by the metal and its concentration at each station.

Metal (0 .005mM)	MRB abundance (cfu g^{-1})		
	Vypin	Munambam	Eloor
Zn	1.1 ± 0.4 x 10^3	6.0± 0.23 x10^3	2.5± 0.13 x 10^4
Cd	5.0 ± 0.14 x 10^2	5.0 ± 0.19 x10^2	1.7 ± 0.17 x 10^4
Ni	1.2 ± 0.32 x 10^3	2.2± 0.52 x10^2	1.4± 0.52 x 10^4
Co	1.5± 0.22 x10^3	2.0 ± 0.5 x 10^2	1.1± 0.33 x 10^4
Cu	4.0± 0.34 x10^2	1.2 ± 0.64 x 10^2	1.6± 0.2 x 10^4
Hg	Nd	Nd	6.3± 0.4 x10^3

Table 3. Abundance of MRB in the sediment at the 3 stations (Nd-Not detected) Values are expressed as average ± SD

Metal (0 .005mM)	MRB abundance (cfu L^{-1})		
	Vypin	Munambam	Eloor
Zn	3.0 ± 0.34 x 10^2	4.0 ± 0.44 x10^2	6.0 ± 0.21 x 10^3
Cd	1.3 ± 0.15 x10^2	2.0 ± 0.21 x10^2	1.8± 0.08 x 10^3
Ni	4.0 ± 0.04 x10^2	1.2 ± 0.08 x 10^2	3.9± 0.60 x 10^3
Co	7.0 ± 0.39 x10^2	1.4 ± 0.05 x10^2	5.6± 0.12 x 10^3
Cu	4.0± 0.54 x10^2	8.0 ± 0.01 x10^2	2.3± 0.36 x 10^3
Hg	Nd	Nd	4.2 ± 0.9 x 10^2

Table 4. Abundance of MRB in the water at the 3 stations (Nd-Not detected) Values are expressed as average ± SD

4.3.2 Multiple Resistances

Out of the 250 MRB, 130 MRB tolerated 5 mM of all 5 metals except Hg. The percentage of resistance among the MRB varied with metal and station. More than 80% of MRB from water and sediment of Eloor showed resistance to Zn, Ni, Cu and Co

(Figure 3a). At Vypin and Munambam, the percentage of MRB resistance to different metals was < 20 and 30 % respectively. MRB resistant to Hg was seen only at Eloor and percentage of Hg resistant MRB was 30 and 20 % in sediment and water, respectively.

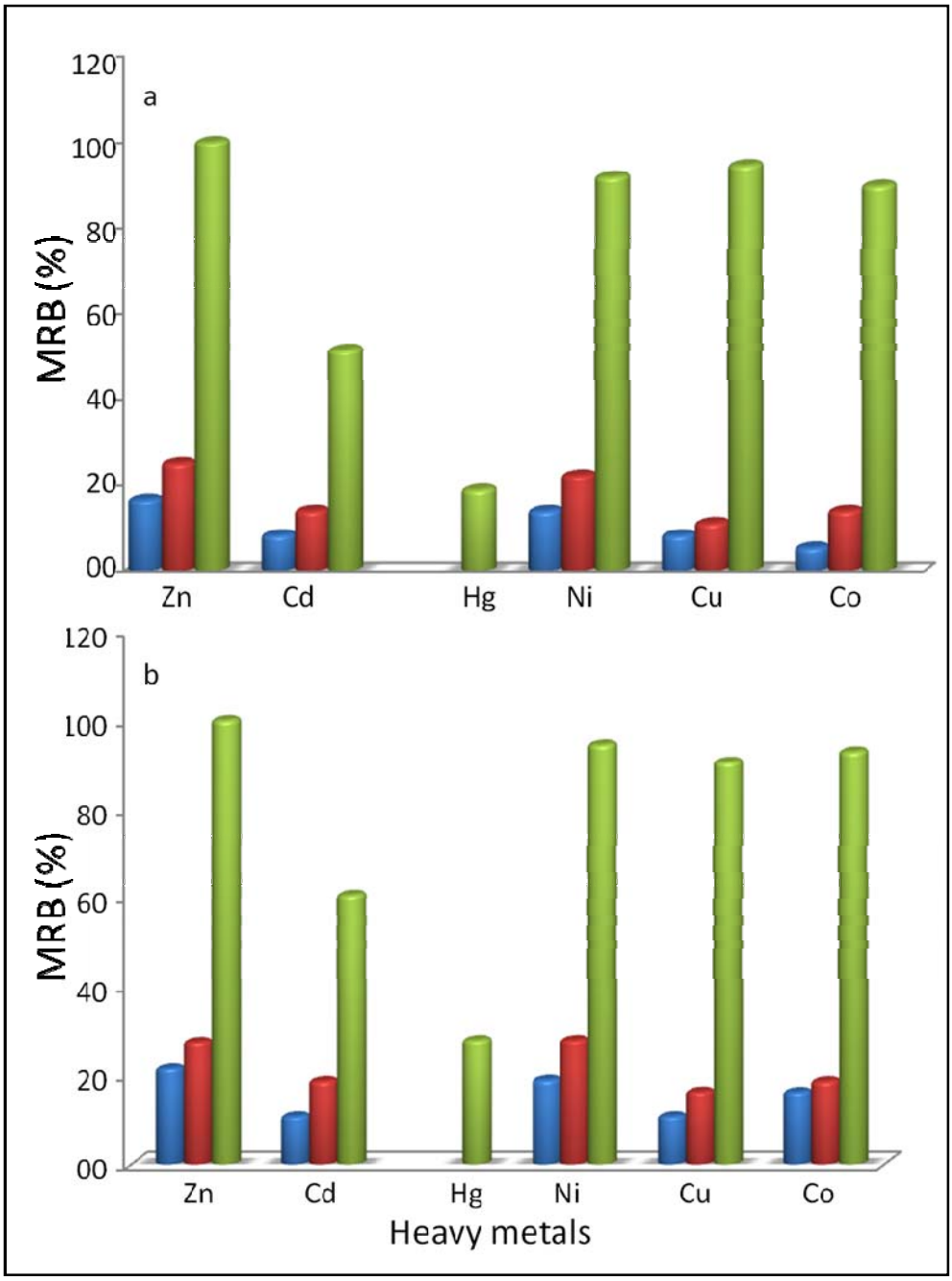


Figure 3a Tolerance of MRB from Vypin( )Munambam( ) and Eloor( ) to different metals (a) Water (b) Sediment

The percentage of multiple MRB in the 3 stations is given in Figure 3b. Multiple MBR to 3 metals was observed in the sediment and water in all the 3 stations. There was difference in the percentage of multiple MBR in water and sediment. The percentage of multiple MBR irrespective of the number of metals was high at Eloor in sediment and water.

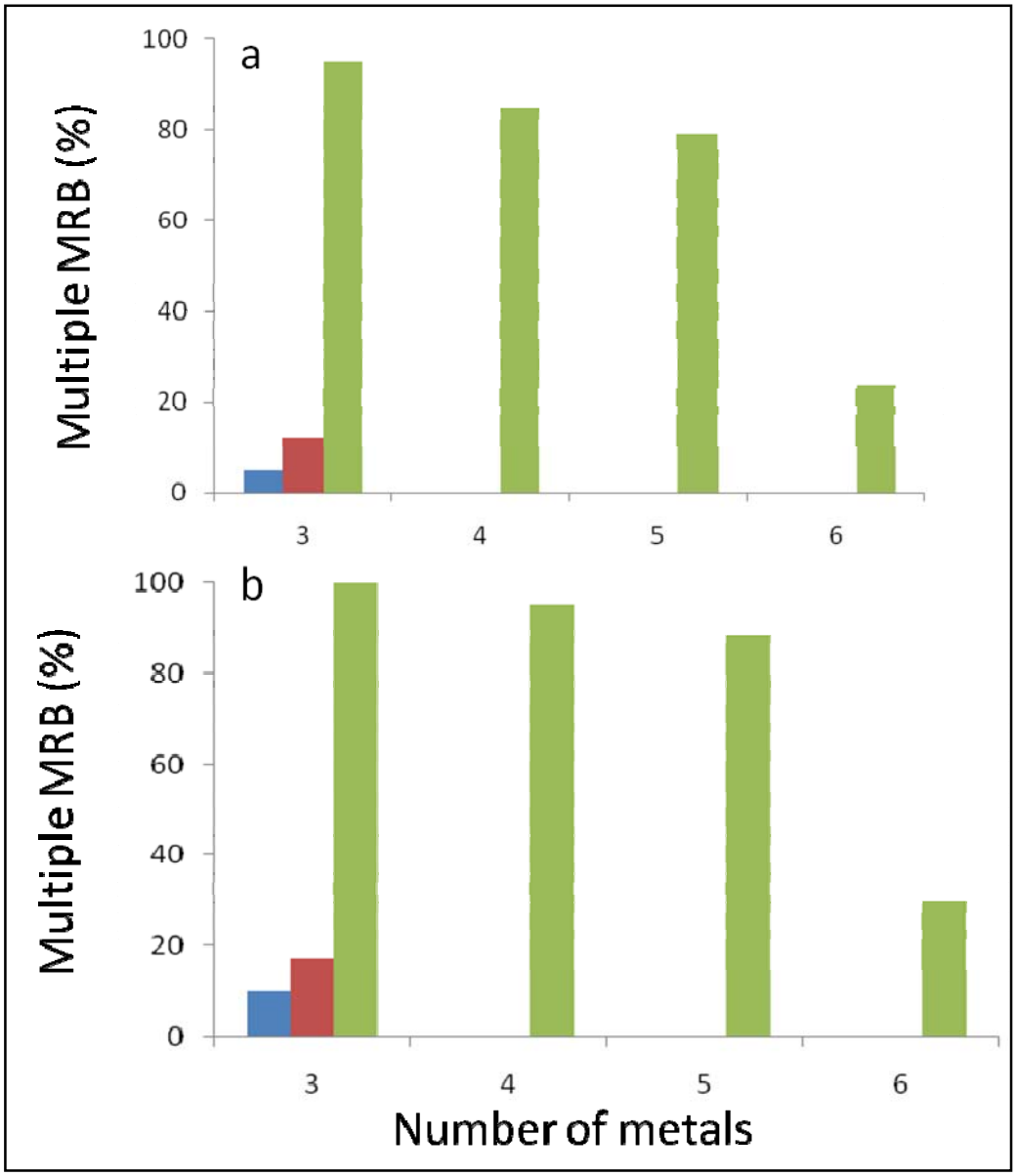


Figure 3b: Percentage of Multiple MRB a) Water b) Sediment at the 3 stations. ( )Vypin ( ) Munambam ( )Eloor

The effect of different concentrations of $AgNO_3$ (0.5 to 1000 mM) on MRB is shown in Figure 4. Eloor recorded higher percentage of resistant MRB compared to the other 2 stations. In 250 mM concentration of AgNO3 the % of resistance was

higher in sediment than water. However, at higher concentration of 500 and 1000 mM, only sediment MRB from Eloor showed resistance (~40%). Bacteria from Vypin and Munambam were the least resistant to $AgNO_3$ with less than 40% showing resistance at 0.5 mM concentration.

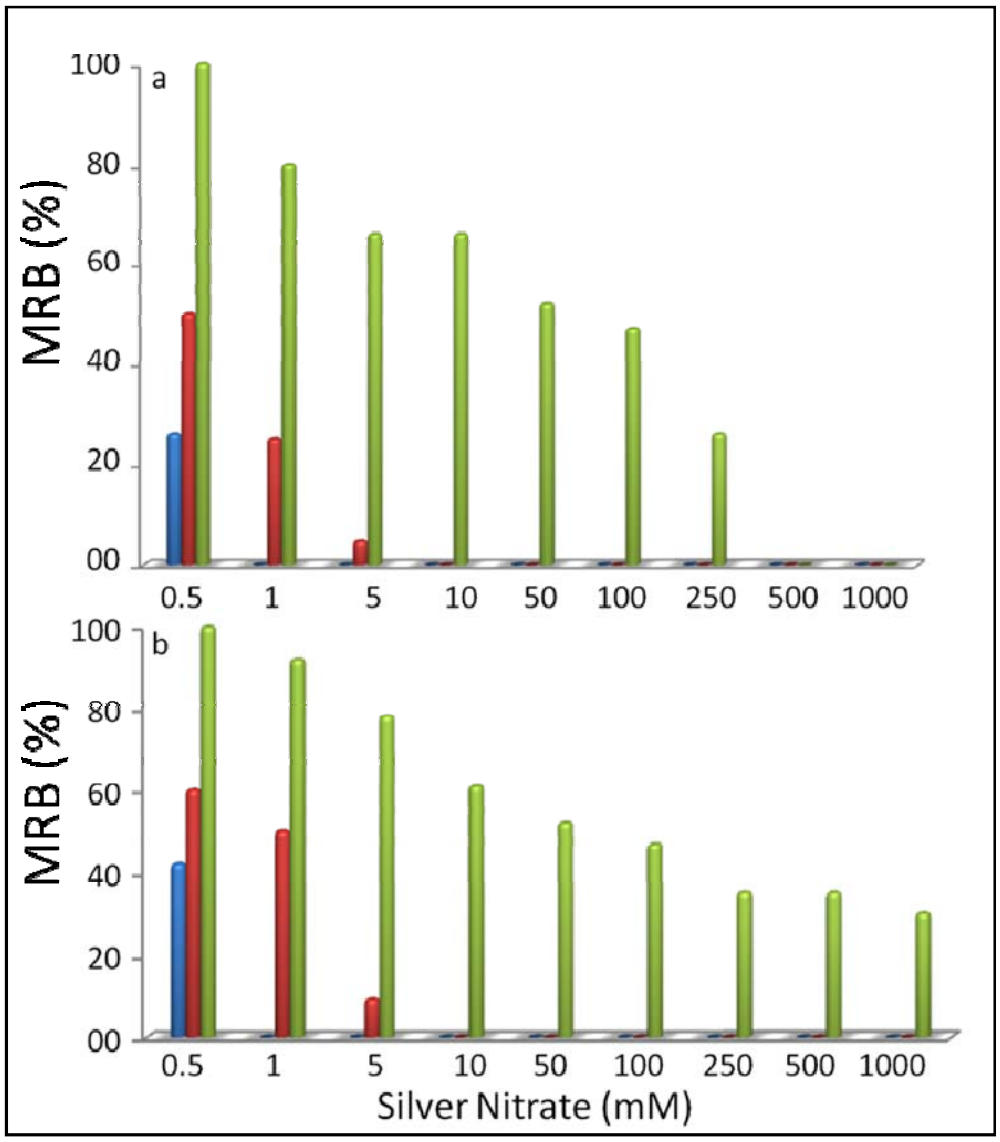


Figure 4: Percentage of silver nitrate resistant MRB at Vypin ( )Munambam ( ) and Eloor( )(a) Water and (b) Sediment

4.3.3. Antibiotic Resistance

Out of 250 MRB, 121 MRB showed resistance to at least one of the antibiotics tested. The number of resistant bacteria in the sediment was more than water (Figure 5), a trend similar to heavy metal. More than 50% of the MRB from Eloor sediment showed

resistance against antibiotics A, At, Ak, C, Cf, Na, Nf, T and Va (expansion given M&M Table 1). Least resistance was for Gentamicin (G) (Protein synthesis inhibitor) and Trimithoprim (Tr) (nucleic acid synthesis inhibitor). The percentage of resistance to antibiotics was higher among MRB from Eloor than Vypin and Munambam. At Vipin, the percentage of antibiotic resistant MRB in the water was less than 10%.

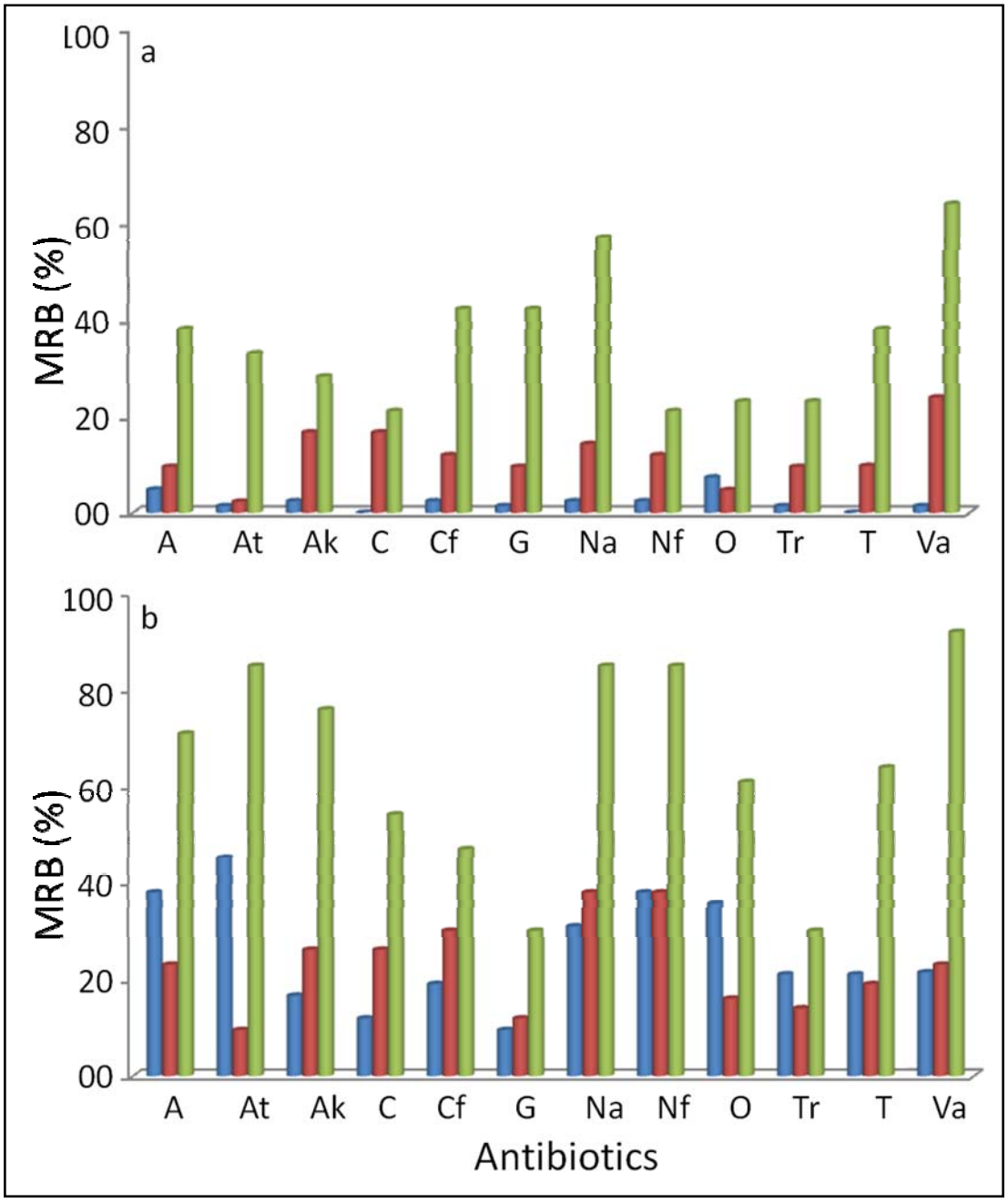


Figure 5. Percentage of antibiotic resistance in MRB at Vypin ( )Munambam ( ) and Eloor( ) (a) Water and (b) Sediment

Approximately 40% of the MRB from Eloor sediment showed Multiple Antibiotic Resistance (MAR) index more than 0.75 (Figure 6). At Vypin and Munambam more than 60% of the MRB from sediment had MAR index below 0.25. MAR index below 0.25 was seen in 80% of MRB from of Vypin water.

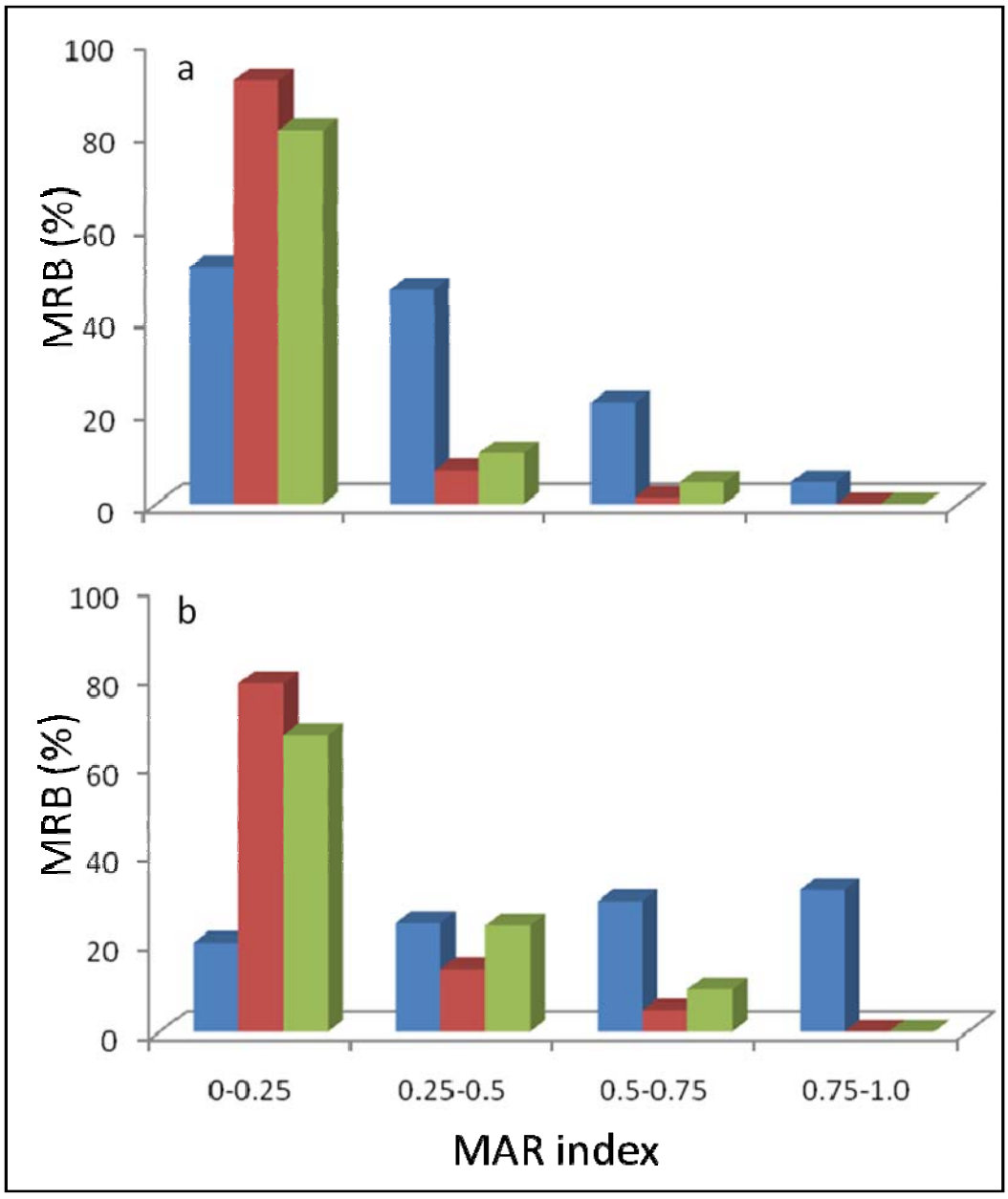


Figure 6. MAR index of MRB at Vypin ( )Munambam ( ) and Eloor( )(a) Water and (b) Sediment

4.3.4 Enzyme expression profile

The enzyme expression of MRB at the three stations is given in Figures 7a. It was observed that all the MRB, irrespective of their source of isolation (water or sediment) expressed 7 hydrolytic enzymes. However, there was variation in the percentage of MRB expressing different enzymes. In general the percentage of MRB with enzyme activities was lower in the water than in the sediment samples. The enzyme expression profile of MRB from Eloor was low (< 20%).

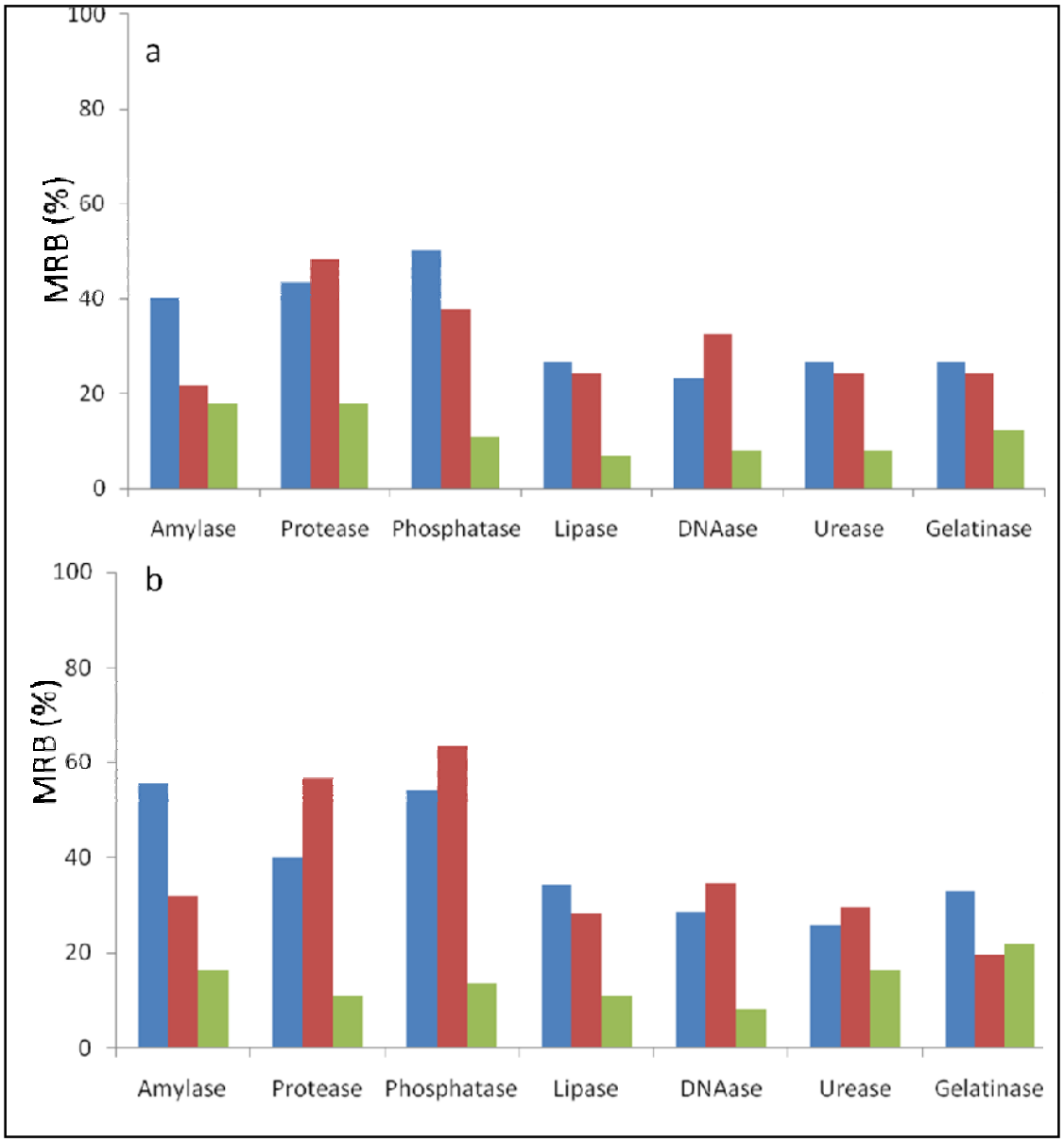


Figure 7a Enzyme expressions of MRB at Vypin ( ) Munambam ( ) and Eloor ( ) (a) Water and (b) Sediment

It was observed that >80% of the MRB from Eloor produced "low level" of protease, amylase, gelatinase and lipase whereas >70% of the MRB from Vypin water showed "medium" production of enzymes (Figure 7b). Munambam water showed more than 40% "medium" production. Enzyme profiling of MRB suggests that the expression in number and extant of enzyme production was higher in the least polluted Vypin station. Thus, it is apparent that metal pollution has a strong impact on the enzyme expression profile of MRB.

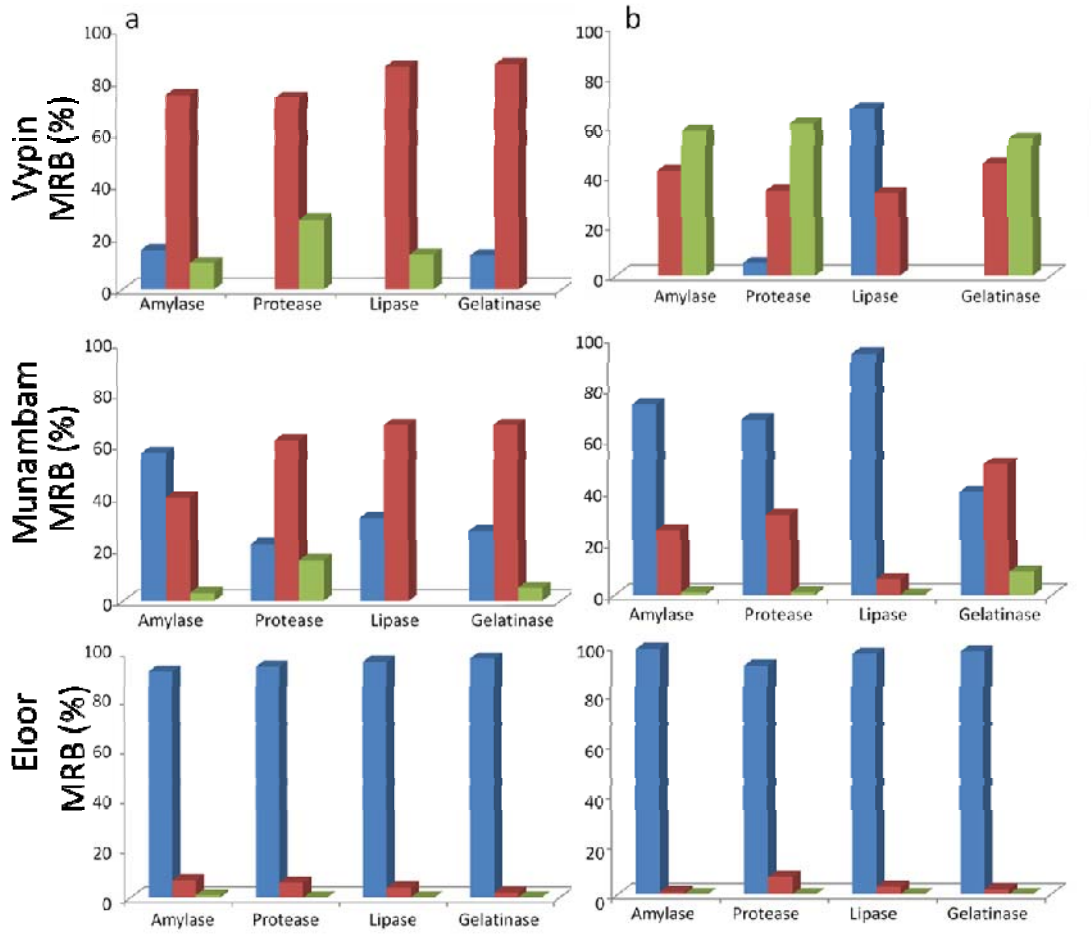


Figure 7b. Percentage of MRB with different level of enzyme production (a) Water (b) Sediment ( ) low( ) medium ( ) high

4.3.5 Identification of MRB

Two hundred and fifty MRB isolates were affiliated to 2 major phyla Proteobacteria and Firmicutes and two minor phyla Actinobacteria and Bacteriodetes (Figure 8). At Eloor Proteobacteria and Firmicutes were present in the

sediment whereas an additional phylum Actinobacteria was recorded in the water. Vypin and Munambam recorded 4 phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes. Actinobacteria and Bacteriodetes were <10% in Vypin and Munambam. In the sediment Proteobacteria was 71, 59 and 48 % at Eloor, Munambam and Vypin respectively. Among the different phyla, Firmicutes was the dominant phylum in the water at Vypin (56.4%) and Munambam (53%) while at Eloor it was Proteobacteria (63%). The number of phyla was low in polluted Eloor.

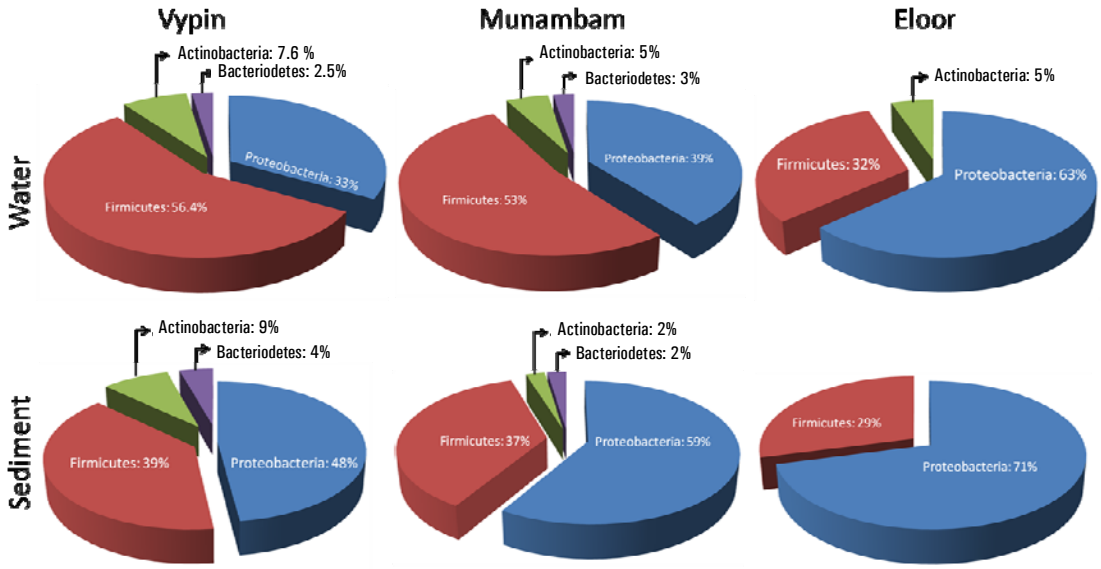


Figure 8. Diversity of MRB in water and sediment at the 3 stations

In CE, 26 genera and 46 species were recorded and the number varied within the phylum and between the stations. There were 21 species of Proteobacteria (Table5), 17 species of Firmicutes (Table 6), 5 species of Actinobacteria (Table7) and 3 species of Bacteriodetes (Table 8). In the sediment 31 species and in water 26 species were recorded. Maximum number of species was recorded at Vypin. Among the 46 species, only 8 species were common in the 3 stations. The common species under phylum Proteobacteria were *Comamonas testosterone, Listonella anguillarum, Pseudomonas aeruginosa, Pseudomonas huttiensis, Shigella flexinari* and *Vibrio furnissi*, *Bacillus filicolonicus* and *Bacillus pumilus* under phylum Firmicutes. Maximum Proteobacteria species (18) were recorded in sediment at Eloor and ,6 species of Firmicutes. The number of Fimicutes species was low at Eloor compared to other two stations. The Firmicutes isolated from Eloor were *B. cereus, B.*

filicolonicus, B. pumilus GC-sub gp B, Staphylococcus cohini, S. aureus and S. *gallinarum*. At Vypin, 17 species of Firmicutes were recorded in the sediment.

Proteobacteria	Vypin		Munambam		Eloor	
	W	S	W	S	W	S
Acinetobacter baumanni	-	+	+	+	-	-
Cedacia davicae	-	-	-	-	-	+
Chromobacterium violacium	+	-	-	-	+	+
Citrobacter koseri	-	+	-	-	-	-
Comamonas acidovorans	-	+	-	-	+	+
Comamonas testosterone	-	+	+	-	+	+
Escherichia coli	+	+	-	-	+	+
Enterobacter gergoviae	-	-	-	-	+	+
Kluyveraascorbata	+	+	-	+	-	-
Listonella anguillarum	+	+	-	+	+	+
Proteus mirabilis	-	-	-	+	-	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Pseudomonas huttiensis	-	+	+	+	+	+
Serratia marcescens	-	+	-	-	+	+
Shewanella putefaciens	-	+	-	-	+	+
Shigella flexnari	+	-	+	+	+	+
Vibrio alginolyticus	-	-	-	-	+	+
Vibrio cholera	-	-	-	+	+	+
Vibrio furnissii	+	-	-	+	+	+
Xanthomonas arboricolapruni	-	+	-	-	+	+
Yersinia pseudo tuberculosis	-	+	-	-	+	+

Table 5. Species distribution of Proteobacteria at 3 stations (W- water;S- sediment)

Firmicutes	Vypin		Munambam		Eloor	
	W	S	W	S	W	S
Bacillus atrophaeus	+	+	+	-	-	-
Bacillus cereus	+	+	-	-	+	+
Bacillus cereus Sub group A	+	-	-	-	-	-
Bacillus filicolonicus	+	+	+	-	+	+
Bacillus marinus	+	-	+	+	-	-
Bacillus mycoides GC-Sub gp	+	+	+	+	-	-
Bacillus oleronius	+		+	+	-	-
Bacillus pumilus GC-Sub gp B	+	+	+	+	+	+
Bacillus sphaericus	-	+	+	+	-	-
Bacillus sp	+	-	-	+	-	-
Bacillus subtilis	+	+	-	-	-	+
Exignobacterium acetylicum	-	+	-	-	-	-
Paenibacillus larvae pulvifaciens	+	+	-	-	-	-
Staphylococcus cohinicohini	+	+	-	-	+	+
Staphylococcus aureus	+	+	-	-	+	+
Staphylococcus gallinarum	+	-	-	-	+	+

Table 6. Species distribution of Firmicutes at the 3 stations (W- Water; S- Sediment) .

Actinobacteria	Vypin		Munambam		Eloor	
	W	S	W	S	W	S
Cellulomonas turbata	-	+	+	-	+	-
Curtobacterium flaccumbaciens	+	+	-	-	-	-
Micrococcus luteus	+	-	+	-	-	-
Micrococcus lylae	-	+	-	-	-	-
Nocardia-nova	+	+	-	+	-	-

Table 7. Species of Actinobacteria at 3 the stations (W- water; S- sediment)

Bacteriodetes	Vypin		Munambam		Eloor	
	W	S	W	S	W	S
Chryseobacterium-balustinum	-	+	-	-	-	-
Flavobacterium	+	-	-	-	-	
Myroidesod oratus	-	+	+	+	-	-

Table 8. Species distribution of Bacteriodetes at the 3 stations (W- Water S- Sediment)

Three way Analysis of Variance (ANOVA) of the Proteobacteria showed significant variation in species between water and sediment samples ($F_{(1,40,5\%)}$ = 5.1511 P<0.05) and also between the three stations ($F_{(2,40,6\%)}$= 2.976, P<0.06) in the first order interaction effect (Table 9).

Source	SS	dof	MSS	Fratio
Between water andsediment (A)	6.2222	1	6.2222	5.1511*
Between stations (B)	7.1905	2	3.5952	2.9764[a]
Between species (C)	34.3810	20	1.7191	1.4231[Ns]
AB interaction	0.01587	2	0.0079	0.006569[Ns]
BC interaction	57.1440	40	1.4286	1.1827[Ns]
AC interaction	8.4440	20	0.4222	0.3495 [Ns]
Error	48.3175	40	1.2079	
Total	161.7550	125		

Table 9.3 Way ANOVA table for Proteobacteria (*P <0.05,$F_{(1,40)}$; a - P < 0.06, $F_{(2,40)}$ NS - P> 0.05 not significant)

It was observed that number of species in sediment at Eloor was significantly different from that at Vypin water ($t_{(40,5\%)}$ = 3.2442, P<0.05) and Munambam water ($t_{(40,5\%)}$ = 2.409,P< 0.05) (Table 10).Based on the spatial distribution sediment of Eloor was found to be favorable for Proteobacteria (Average abundance is Maximum = 1.619) whereas water and sediment of Vypin are least favorable and species of this group were with highest variability in the water samples.

Proteobacteria	Vypin Water	Munambam water	Eloor water	Vypin sediment	Munambam sediment	Eloor sediment
Vypin Water	-	○	●	○	○	●
Munambam water	0.268	-	○	○	○	●
Eloor water	1.976	1.257	-	○	○	○
Vypin sediment	1.508	0.936	0.359	-	○	●
Munambam sediment	1.391	0.989	0.000	0.253	-	○
Eloor sediment	3.244	2.409	1.637	1.854	1.206	

Table 10. Trillis diagram showing the significance of the difference between water and sediment samples (○- Calculated t is not significant at 5% P>0.05. ●- Calculated t is not significant at 10% P<0.06. ●- Calculated t is significant at 5% P<0.05 for 40 degrees of freedom)

The 3 way Analysis of Variance (ANOVA) of species of phylum Firmicutes revealed that there was no significant difference in water and sediment samples (P> 0.05). But between stations the abundance of species vary significantly ($F_{(2,32,14\%)}$ = 2.0942,P<0.14). (Table11). Further the species of this group shows a selective preference for the locations as indicated by high station –species interaction ($F_{(32,32,5\%)}$ = 2.412,P<0.05).

Source	SS	df	MSS	F ratio
Between water and sediment (A)	0.3529	1	0.3529	0.4308 ^{NS}
Between stations (B)	3.4314	2	1.7157	2.0942 ^{b}
Between species (C)	25.2941	16	1.5809	1.9297^{a}
AB interaction	1.1176	2	0.5588	0.6821 ^{Ns}
BC interaction	63.2352	32	1.9761	2.4121*
AC interaction	10.3146	16	0.6446	0.7868 ^{Ns}
Error	26.2157	32	0.8192	
Total	129.9615	101		

Table 11. 3way ANOVA table for Firmicutes (*significant P< 0.05, $F_{(32,32)}$; a- significant P < 0.06 calculated $F_{(32,32)}$; b significant - P< 0.14, $F_{(32,32)}$)

There was significant difference in Firmicutes species in water between Eloor and Vypin ($t_{(32,5\%)}$ = 2.0383,P<0.05). No such difference was seen sediment at the 3 stations (P>0.05) (Table 12).

Firmicutes	Vypin Water	Munambam water	Eloor water	Vypin sediment	Munambam sediment	Eloor sediment
Vypin Water	-	○	●	○	○	○
Munambam water	0.307	-	○	○	○	○
Eloor water	2.038*	1.311	-	○	○	○
Vypin sediment	0.754	0.295	1.223	-	○	○
Munambam sediment	1.084	0.647	0.587	0.445	-	○
Eloor sediment	1.259	0.785	0.447	1.603	0.132	-

Table 12. Table 12 Trillis diagram showing the significance of the difference between water and sediment samples (○- Calculated t is not significant at 5% P>0.05. ●- Calculated t is significant at 5% P<0.05 for 32 degrees of freedom).

Shannon Weiner index (H') was higher in sediment than water. Vypin, recorded the highest H' of 3.1 and 3.3 in water and sediment, respectively. In Eloor H' index was 2. 8 and 2.7 in sediment and water, respectively. The diversity decreased with increase in metal concentration. The species diversity index (H') at the three stations is presented in Table 13. At Eloor maximum species diversity was observed among Proteobacteria (2.70 in water and 2.89 in sediment, respectively) whereas the diversity index (H') was low for phylum Firmicutes (1.71 in water and 1.72 in sediment).

Phylum	Vypin		Munambam		Eloor	
	Water	Sediment	Water	Sediment	Water	Sediment
Proteobacteria	1.94	2.56	2.07	2.48	2.70	2.89
Firmicutes	2.60	2.37	2.05	1.73	1.72	1.71
Actinobacteria	1.09	1.38	0.69	ND	ND	ND

Table13. Shannon Weiner Indices (H') in the 3 stations (ND-not detected)

Cluster analysis based on species showed clear separation between stations irrespective of the samples (Figure 9). More than 80% similarity in the diversity was observed among the MRB from Eloor water and sediment whereas there was less than 60% similarity between Eloor and Vypin. The clustering based on metal concentration was similar to that of species diversity, clearly reflecting the influence of metal concentration on diversity.

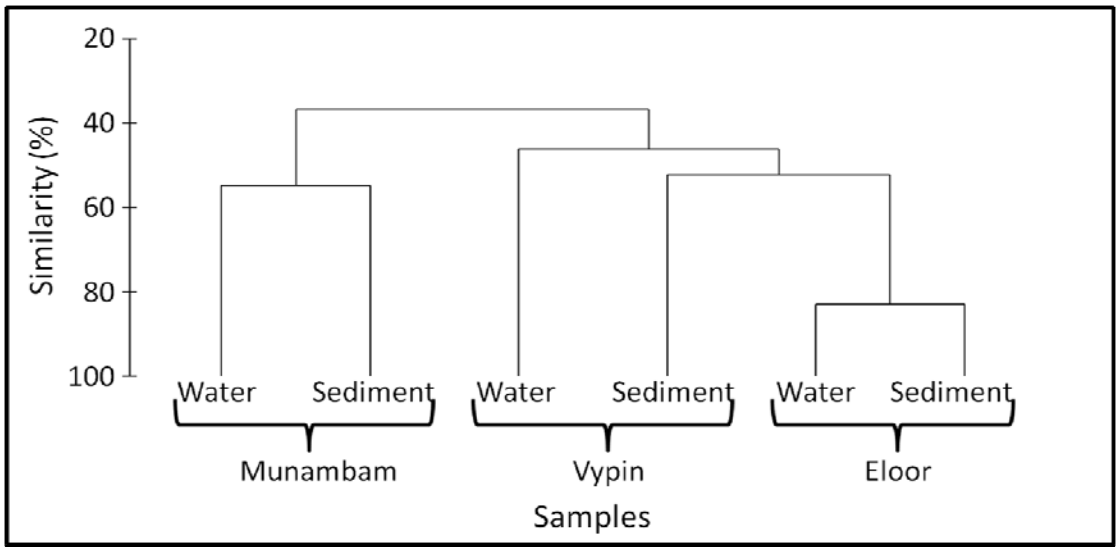


Figure 9. Cluster analysis of water and sediment samples from the three stations. Bacterial diversity was used to associate the stations.

4.4. Studies on "MDR" pathogenic MRB

The multiple metal and antibiotic resistance profiles of five selected pathogenic bacteria are given in Table 14 a, 14b and 14c. *V. alginolyticus* showed maximum MAR index of 1.0, i.e resistant to all the antibiotics tested, followed by *E. coli* (MAR index 0.91), *P. aeruginosa* and *B. subtilis* (MAR index 0.75) and *S. aureus* (MAR index 0.25). Interestingly *S. aureus* showed *higher* tolerance to $AgNO_3$ (1000mM) and *B. subtilis* showed lowest tolerance (100mM). Gram negative isolates, *E. coli, V. alginolyticus* and *P. aeruginosa*, showed tolerance up to 500 mM concentrations of $AgNO_3$. All the cultures could tolerate upto 5mM concentration of other studied metals except *B.subtilis* (2.5mM).

Bacterial species	MARindex	$AgNO_3$ concentration
B. subtilis	0.75	100 mM
S. aureus	0.25	1000 mM
E. coli	0.91	500 mM
V. alginolyticus	1.00	500 mM
P. aeruginosa	0.75	500 mM

Table14a. MAR index and $AgNO_3$ tolerance level of MDR pathogenic MRB

Bacterial species	Zn	Cd	Hg	Ni	Cu	Co
B. subtilis	5mM	5mM	2.5mM	5mM	5mM	5mM
S. aureus	5mM	5mM	5mM	5mM	5mM	5mM
E. coli	5mM	5mM	5mM	5mM	5mM	5mM
V. alginolyticus	5mM	5mM	5mM	5mM	5mM	5mM
P. aeruginosa	5mM	5mM	5 mM	5mM	5mM	5mM

Table14b. Metal tolerance among MDR pathogenic MRB

Antibiotics	*S.aureus*	*B.subtilis*	*E.coli*	V.alginolyticus	*P.aeruginosa*
A	R	R	R	R	R
At	S	R	R	R	R
Ak	R	S	R	R	S
C	S	R	R	R	R
Cf	S	S	R	R	S
G	S	R	S	R	R
Va	S	R	R	R	R
Na	R	R	R	R	R
Nf	S	R	R	R	R
O	S	R	R	R	R
Tr	S	R	R	R	R
T	S	S	R	R	S

Table14c. Antibiotic resistance among MDR pathogenic MRB

4.4.1 Effect of AgNPs

The effect of different concentrations of AgNPs on MDR pathogenic MRB is shown in Figure 10. All the Gram negative microorganisms, *E. coli*, *P. aeruginosa* and *V. alginolyticus*, were sensitive to AgNPs and the level of sensitivity varied between organisms. *P.aeruginosa* showed resistance up to 60 µg ml^{-1}AgNPs and complete mortality of cells occurred at 80 µg ml^{-1}. *V. alginolyticus* was highly sensitive with complete mortality even at lowest concentration tested (20 µg ml^{-1}) while more than 40 % of *P. aeruginosa* cells were resistant up to 60 µg ml^{-1}. Negligible percentage of *E. coli* cells (<10 %) were resistant up to 100 µg ml^{-1}AgNPs. Gram positive bacteria also showed differences in their resistance pattern against AgNPs. *B. subtilis* was highly sensitive and complete mortality was observed at minimum concentrations (20 µg ml^{-1}), while approximately 80 % of *S. aureus* cells were resistant to 100 µg ml^{-1}AgNPs.

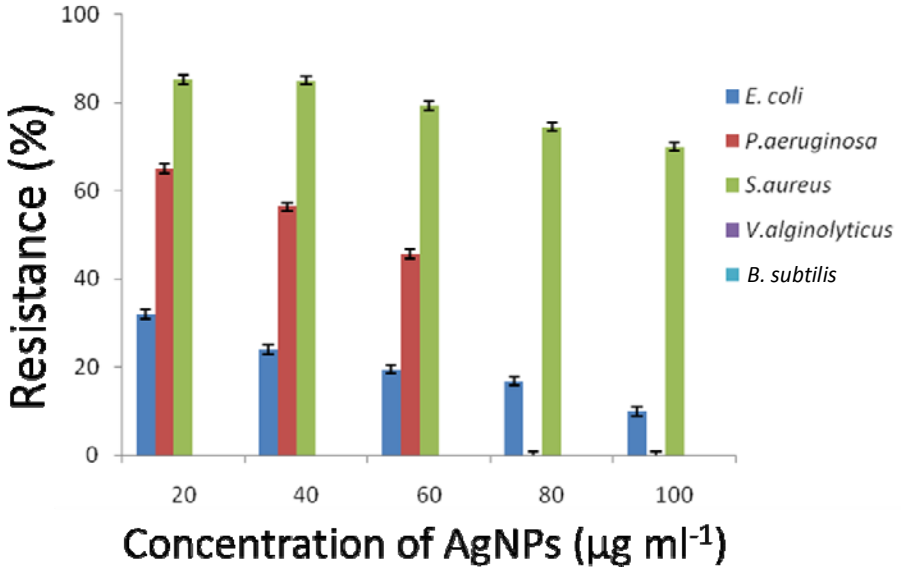


Figure10. Percentage of resistance of MDR pathogenic MRB to AgNPs Values are expressed as average ± SD

4.4.2 Cell wall Integrity

The cell wall integrity of test organisms on exposure to AgNPs was studied using Scanning electron microscopic and SDS assay. The morphology of the cell of Gram negative MRB affected after AgNPs treatment. The damage was more severe in *E. coli* and *P. aeruginosa*, as the surface of majority of cells became rough and elongated and cell wall breakage was seen in the SEM images (Figure 11). In the case

of Gram positive *B. subtilis* the cell surface was distorted but in *S. aureus* there was no significant changes in the morphology of the cell.

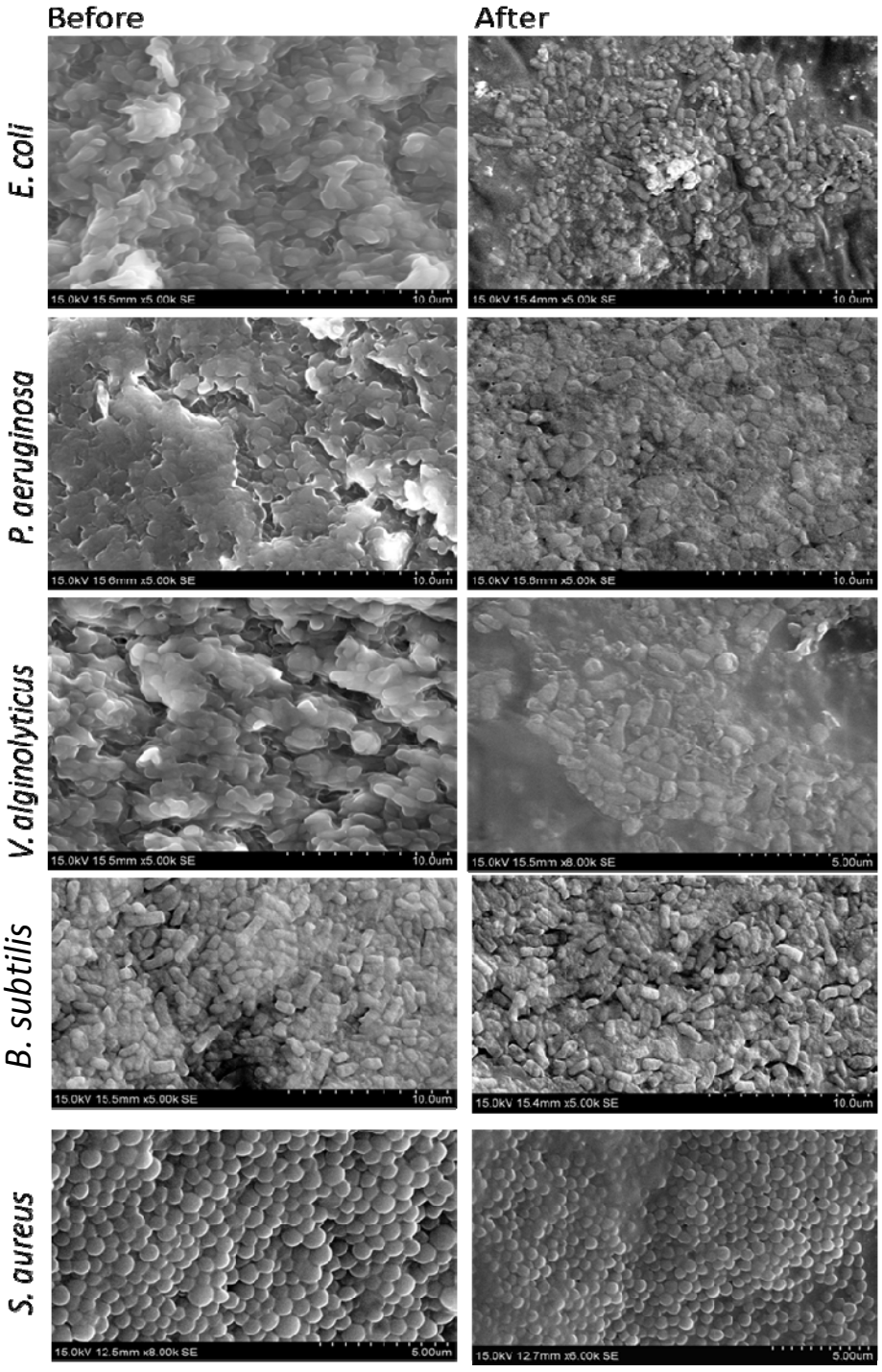


Figure11 Scanning electron Micrographs depicting the effect of AgNPs on the bacterial cell morphology at a magnification of 5000X. Before is without and after is with AgNPs treatment

The cell wall integrity was quantified based on SDS assay. The percentage of cell wall integrity of 5 MDR pathogenic MRB is shown in Figure 12. The cell wall integrity of Gram negative strains *V. alginolyticus*, *P. aeruginosa* and *E. coli* was reduced drastically after 50 minutes of incubation with AgNPs. The cell wall of *B. subtilis*was more resistant to AgNPs with only <20% loss of cell wall integrity in 50 minutes and approximately 40 % loss of integrity time after 90 minutes. The cell wall of *S. aureus* was highly resistant to AgNPs and maintained more than 75% of its cell wall integrity even after 120 minutes of exposure in 100 µg ml^{-1}AgNPs .

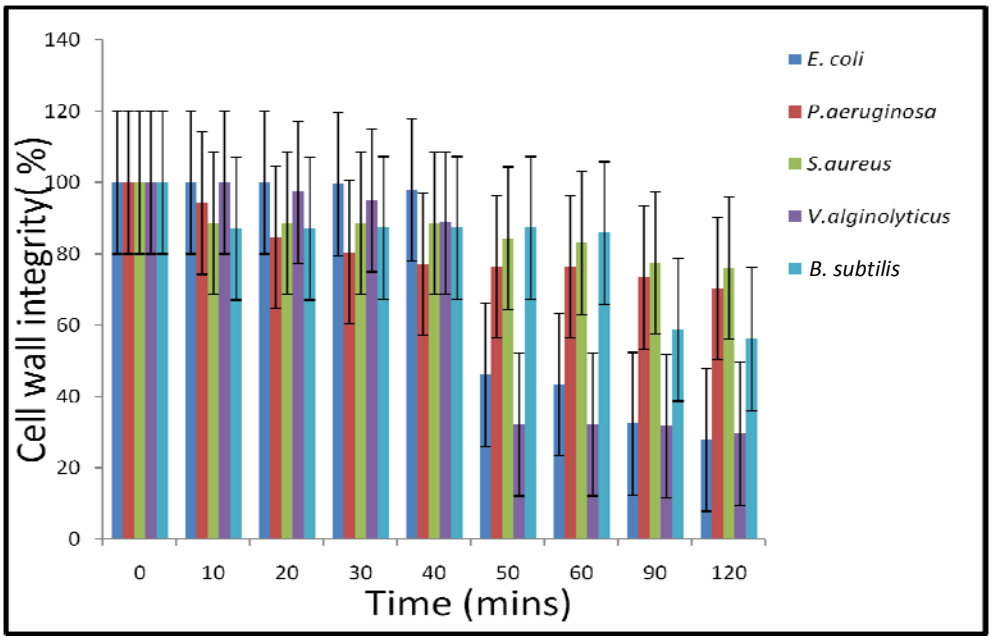


Figure 12. Cell wall integrity of MDR pathogenic MRD after exposure to AgNPs.

4.4.3 Metabolic activity of cells

Metabolic activity of the test organisms decreased with increasing concentrations of AgNPs except for *S. aureus* (Figure 13). The ratio of metabolically active to total cells in *P. aeruginosa*, *V. alginolyticus* and *B. subtilis* started decreasing on exposure to 20 µg$ml^{-1}$and reached a minimum of 0.2 at 60 µgml^{-1}AgNPs. *E. coli* cells were metabolically active upto 40 µgml^{-1} and minimum activity reached at 60 µgml^{-1} of AgNPs. Interestingly no significant changes in the metabolic activity of *S. aureus* were observed even at highest (100 µgml^{-1}) concentrations of AgNPs.

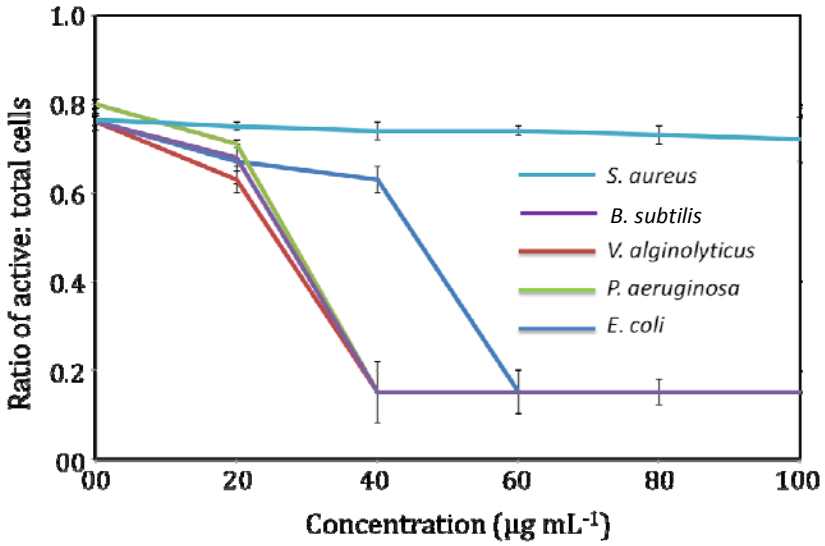


Figure 13. Effect of AgNPs on metabolic activity of MDR pathogenic MRB. Metabolic activity is expressed as ratio of active to total cell .Values are expressed as average ± SD

4.4.4 Cell wall composition

There was quantitative and qualitative change in the whole cells fatty acid composition of the sensitive Gram positive and negative multiple drug resistant MRB when treated with AgNPs. In the case of Gram negative *E. coli*, fatty acids concentration of $C_{17:0}$cyclo reduced from 25% in control to 12 % in treated cells whereas the concentration of $C_{16:00}$ increased from 7 % in the control to 35 % in the AgNPs treated cells. In *P. aeruginosa* cells the concentration of the fatty acids such as 10:0 3OH, 12:0 3OH17:0 cyclo and 19:0 cyclo w8c were affected. *V. alginolyticus* fatty acids such as $C_{16:00}$ and $C_{14:00}$ decreased and $C_{12;00}$ increased (Table 15).

Fatty Acid	*E. coli*		*V. alginolyticus*		*P. aeruginosa*	
	Fatty acid (%)		Fatty acid (%)		Fatty acid (%)	
	Experiment	Control	Experiment	Control	Experiment	Control
12:00	4.41	2.43	8.61	3.77	3.62	3.96
14:00	8.59	8.02	4.6	8.2	--	-
17:0 cyclo	12.98	25.46	0.61	-	1.68	2.21
16:00	34.96	7.19	24.53	31.18	35.08	33.91

Table 15. Percentage of fatty acid composition of gram negative "MDR" pathogenic MRB (Experiment represents AgNPs treated cultures: Control is untreated)

In both Gram positive cultures (*B. subtilis and S aureus*) the level of 15 antiiso fatty acid concentrations declined after the treatment with AgNPs whereas 15 iso and C $_{17:\ 00}$ concentrations increased and it was very prominent in *S.aureus* where the concentration of 15 iso fatty acid increased from 10.25 % in control to 53.1 % in AgNPs treated cells (Table 16). The chromatogram of fatty acid composition of the 5 MDR bacteria is given in Appendix

Fatty acid	*B subtilis*		*S aureus*	
	Fatty acid (%)		Fatty acid (%)	
	Experiment	Control	Experiment	Control
12:00	0.1	0.26	-	0.16
14:0 iso	1.19	1.12	1	1.56
15:0 iso	55.02	52.9	53.1	10.25
15:0 anteiso	19.71	23.81	19.93	44.81
16:0 iso	3.35	2.27	3.17	2.13
16:00	3.88	2.88	3.47	3.75
17:0 iso	9.87	5.84	10.75	7.83
17:0 anteiso	4.44	4.01	5.29	13.84

Table16. Percentage of fatty acid composition of gram positive pathogenic "MDR" MRB (Experiment represents AgNPs treated cultures: Control is untreated)

4.4.5 DNA damage

The effects of AgNPs on the integrity of DNA in test organisms were represented as a function of tail length in Comet assay. Figure 14 shows the histogram of comet assay results of MDR strains exposed to 100 µg ml^{-1} concentration of AgNPs. The DNA damage induced by AgNPs was high in *E. coli* and *P. aeruginosa*, wherein more than 45 and 65 % cells, respectively were damaged. The extant of DNA damage was less in *B. subtilis* and *V. alginolyticus*,(ca 15%), However, the cell of *S. aureus* were not affected by AgNPs *as* most of the cells (98%) were under low or no damage class in comet assay. Figure 15 shows is a representative image of heavy damaged and intact / Low damaged DNA of multiple drug resistant pathogenic MRB.

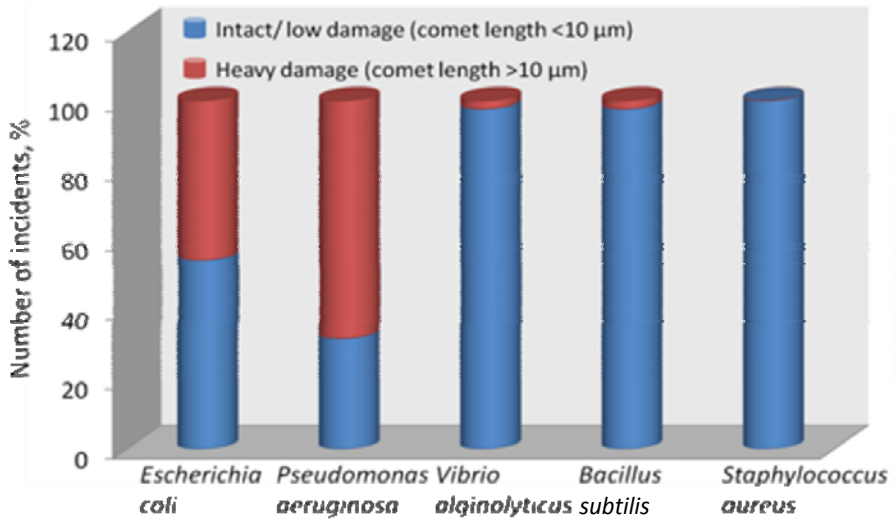


Figure 14. Percentage of DNA damage incidents in MDR pathogenic MRB

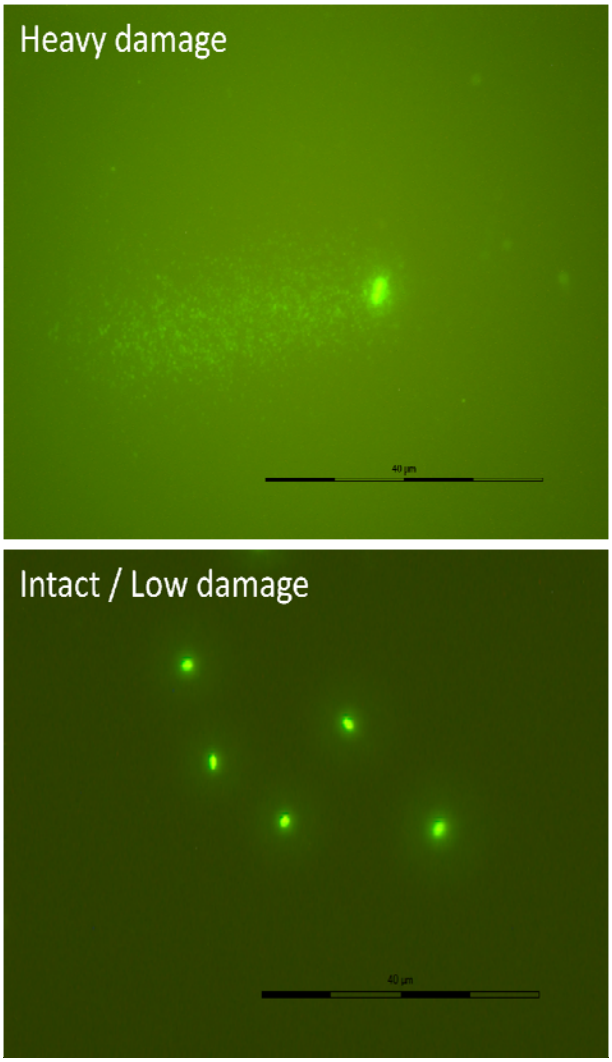


Figure15. Image of heavy damaged and intact/Low damaged DNA of a MDR pathogenic MRB

4.5 Mechanisms of resistance of *S. aureus to* AgNPs

To understand the resistance mechanism of *S. aureus* to AgNPs , the cells were exposed to efflux pump blocking agent Verapamil before treatment with AgNPs. It is observed that none of the cells were resistant to AgNPs in the presence of Verapamil, while more than 80 % were resistant to 100 μgml^{-1} AgNPs in the absence of Verapamil (Figure 16a). The role of efflux pump in rendering AgNPs resistance to *S. aureus* was further confirmed with comet assay (Figure 16,b). DNA damage was observed among more than 60 % of cells in the presence of Verapamil. Figure 17 is a schematic representation of AgNPs resistance mechanisms of *S. aureus*.

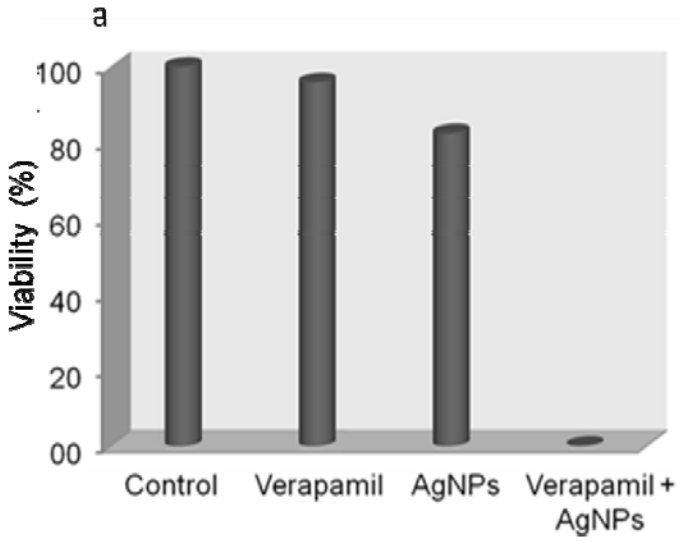


Figure 16a. Percentage of viabilty of *S.aureus* cells to AgNPs after Verapamil treatment

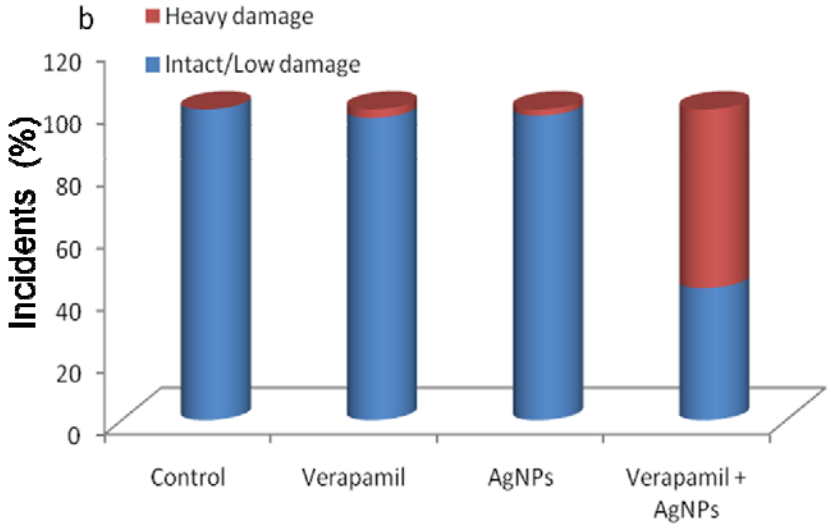


Figure 16b. Percentage of incidents of *S.aureus* cells to AgNPs after Verapamil treatment

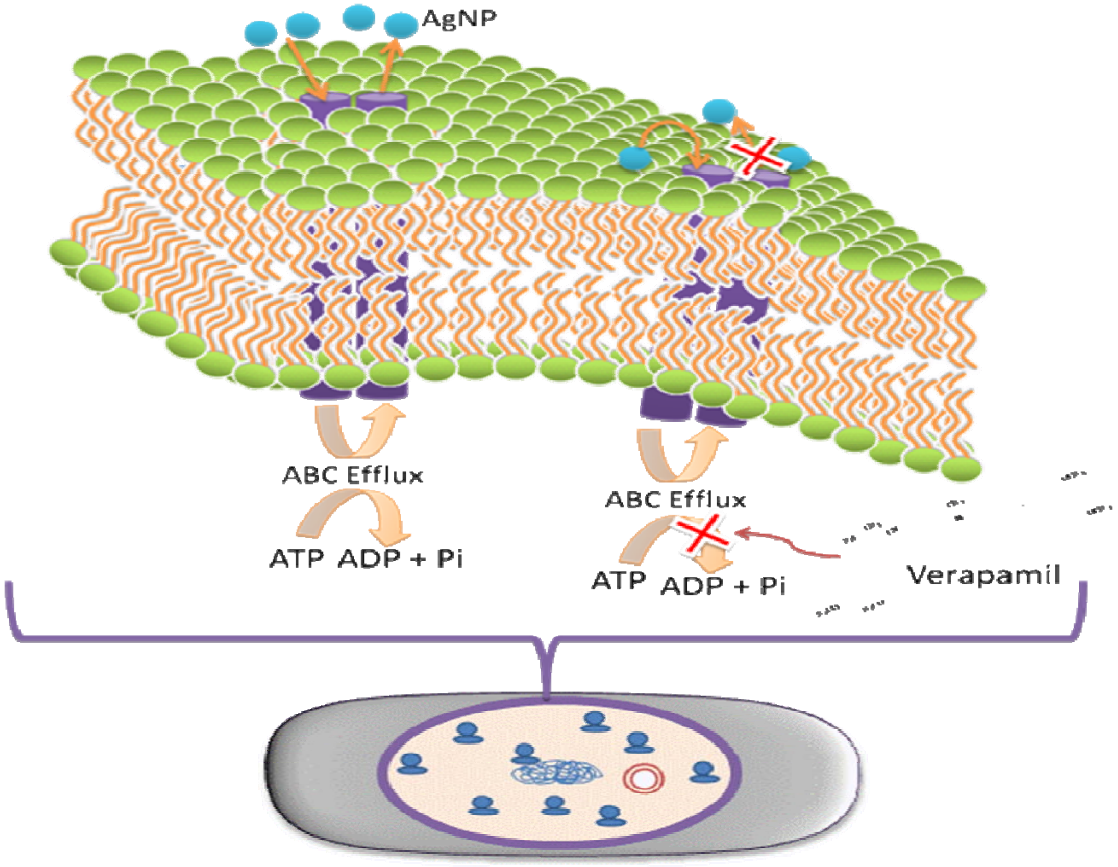


Figure 17. Schematic representation of the AgNPs resistance mechanisms of *S. aureus*

Chapter 5
DISCUSSION

Contents

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 (Balachandranet 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; Sahuand Bhosale,1991; Ram et al., 1998). An increase in

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.,

2007). 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). Azamet 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 $10^{2-4} g^{-1}$ 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

2003). 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 $1/100^{th}$ 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 (Positra, 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 (Takamuraet 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

330 mg Co kg^{-1}) of Oregon soils (Abou-Shanab et al., 2007). 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 (Paulssonet 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 $AgNO_3$.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 $AgNO_3$ only up to a concentration of 1 mM. Bacteria from Vypin and Munambam were the least resistant to $AgNO_3$ 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 *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 andGocke 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 amiinopeptidase 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

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 was 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 e 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, where as 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* losttheir 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 consequentlytheir 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 μgml^{-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 there by 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 *S.aureus*was resistant to AgNPsupto 100µ 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 *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 *E. coli* than *S. aureus*. Stress conditions can regulate the membrane fluidity and stability by adjustments of membrane lipid or fatty acid composition (Navarilzzo et al., 1993). There was increase in 17:0 and 15:0 iso-fatty acid in the resistant *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 *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 μgml^{-1} 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 μgml^{-1}) 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.

Chapter 6
SUMMARY AND CONCLUSION

The thesis entitled "Studies on metal resistant bacteria in Cochin estuary and its response towards antibiotics and silver nanoparticles" is the first study that deals with the metal –microbe interaction in a heavy metal polluted Indian estuary.

Scope

Estuaries are buffer zones with one end opening to the ocean and the other end receiving fresh water. It is also the final repositories for runoff pollutants including metals that are introduced into the system due to anthropogenic activities from the urban and industrial areas. The direct impact of heavy metal pollution on microbial ecosystem includes the alterations in the abundance, physiology and diversity which will have an adverse effect on several biogeochemical cycles of the coastal waters. Understanding the effect of trace metals in heavy metal polluted estuaries is a major goal for the microbial ecologists. In recent years, due to inputs from industries, sewage and excessive use of pesticides and fertilizers, the Cochin estuary (CE) is now categorized as a heavy metal polluted estuary. Comprehensive information on the adaptation/response for bacterial tolerance towards high concentrations of heavy metals in either aerobic or anaerobic marine environments around India is limited. Studies on metal - microbe interaction are hitherto not undertaken in this estuary. This study hypothesize that long-term heavy metal pollution will affect the abundance, diversity and activity of the bacterial community and this pattern also persists along the metal concentration gradient in the estuary.

Accordingly, the following objectives have been proposed for the study:

1. Distribution and enzyme expression profile of metal resistant bacteria along a pollution gradient in the Cochin Estuary.
2. Differential response of metal resistant bacteria towards antimicrobial agents.

3. Mechanisms of metal resistant bacteria towards silver nanoparticles.

Approach

Three stations with varying levels of heavy metal contamination in estuary were selected .viz. highly polluted Eloor, intermediate polluted Munambum and least polluted Vypin, to understand the effect of metal concentration on the bacterial parameters. In this study two common microhabitats viz. sediment and near-bottom water were selected to represent different degree of metal exposure and bacterial density. Sediment harbours higher bacterial densities than water column due to the availability of organic matter. It is also the site of high metal deposition compared to water and hence will be conducive for the selection of resistant genes. These two habitats will indicate which microhabitat is likely to be the favourable source of resistance.

The physico-chemical parameters of the water (temperature, salinity, pH, DO, nitrite, nitrate, phosphate) and metal concentration (Zn, Hg, Co, Cd, Cu, Ni) in both water and sediment were measured using standard techniques. Bacterial variables (TC, TVC, TCC) in water and sediment were enumerated and the composition of metal resistant bacteria (MRB) was studied using MIS. The response of heavy metal resistant bacteria to antibiotics and silver nanoparticles were studied using standard method. The data were processed using the software Primer 6.

Salient Findings:

- The near-bottom water characteristics of the 3 stations were not very different from the earlier reported values of the CE. Station Eloor being close to the Periyar River and near the estuary's headwaters, showed fresh water influence with low salinity of 19 and high nitrate of19.35 µmol $N\text{-}NO_3^-$ L^{-1}.
- The concentration of all five metals was high in the CE. There was a gradient not only in the concentration of metals but also in the number of metals recorded at the 3 stations. Heavy metal concentration was in the order of Zn > Ni > Cd >Cu >Co at all the stations. Station Eloor being close to industrial belt, recorded the highest concentration of all the metals both in sediment and

water whereas at Vypin which is located near the mouth of the estuary had low concentration of all the metals studied. The source of heavy metals in the CE is mostly from industrial and domestic wastes. The 3 way ANOVA showed significant difference in metal concentration between water and sediment ($F_{(1,10)} = 5.144$ $P<0.05$), between metals ($F_{(6,10)} = 7.784$, $P<0.05$) and between stations ($F_{(2,10)} = 10.01$ $P<0.05$).The tidal forcing and river water flow brings about a gradient in metal concentrations in the CE.

- Total bacterial counts in the sediment was 10^{9-10} cells $^{g-1}$and in water it was 10^{8-9} cells $^{L-1}$ at the three stations. It was in the same order as reported for pristine estuaries. However, the culturable fraction was affected by metal concentration. TCC was low in polluted Eloor station ($6.3 \pm 0.5 \times 10^3$ cells $^{L-1}$) compared to the least polluted Vypin ($1.3 \pm 0.13 \times 10^4$ cells L^{-1}).
- MRB population in the sediment ranged between 10^{2-4}cfu g^{-1} and in water it was 10^{2-3}cfu L^{-1} . MRB population at Eloor was high and ranged between $2.5 \pm 0.13 \times 10^4$ and $6.3 \pm 0.4 \times 10$ cfu g^{-1} in sediment and $6.0 \pm 0.21 \times 10^2$ and $4.2 \pm 0.9 \times 10^3$ cfu L^{-1} in water. At Vypin and Munambam, the MRB was one order less than that at Eloor, and was 10^3cfu g^{-1} and 10^4 cfu L^{-1}in sediment and water, respectively.
- Bacterial resistance to different metals increased along the metal contamination gradient in the CE. Percentage of multiple metals resistance was high at Eloor (~80%) compared to Vypin (>20%) and Munanbum (~30%).Resistance was more predominant in sediment than in water at each station due to higher metal bioavailability in the sediment.
- Antibiotic tolerance among MRB showed the same trend. Approximately 40% of the bacterial isolates from Eloor sediment showed Multiple Antibiotic Resistance (MAR) index of above 0.75while more than 60% of the isolates from Vypin and Munambam sediment showed MAR index of below 0.25.This supports the hypothesis that metal contamination directly selects the metal tolerant bacteria and co-selecting the antibiotic tolerance. Majority of the metal and antibiotic MRB were pathogenic.

- The level of heavy metal pollution exerted a reduction/ adaptation in the diversity and enzyme expression profile of MRB.
- The MRB of water and sediment expressed 7 hydrolytic enzymes. There was variation in the number and extent of expression with respect to station. It was observed that >80% of the organisms retrieved from Eloor region had low level of protease, amylase, gelatinse and lipase enzyme expression compared to Munanbam and Vypin. This variability in enzyme expression profile may have been brought about either due to the suppression of the expression of certain enzymes of the same bacteria occurring at different stations or that the heavy metals actually selected different bacteria with different enzyme expression profile.
- There was spatial variation in the diversity of MRB, the Gram negative MRB being more prevalent (64.2%) and increased with the level of metal pollution. MRB were affiliated to Phyla Firmicutes, Proteobacteria, Actinobacteria and Bacteriodetes. The number of phyla decreased with increased metal concentration. A total of 26 genera and 46 species were recorded from the CE and the number varied within the phylum and between the stations. Eloor showed the lowest Shannon Weiner index (H') of 2.8 and 2.7, in sediment and water, respectively. Proteobacteria was the major group of MRB and the percentage increased with the level of pollution. At Eloor, Proteobacteria accounted for 71% of the MRB compared to less polluted Vypin (48 %) and Munambam (59 %). %). Among the different phyla, Firmicutes was the dominant phylum in the water samples of Vypin (56.4%) and Munambam (53%). The 3 way Analysis of Variance (ANOVA) of Proteobacteria showed significant difference between water and sediment, between the three stations and between the species. However, such a significant difference was observed for Firmicutes only in the water of Eloor and Vypin.
- AgNPs has emerged as an alternative therapy to control the multiplication of multiple drug resistant (MDR) bacteria. This is the first study on the effect of AgNPs on marine MDR pathogenic bacteria. MDR pathogenic MRB followed differential response to AgNPs. Gram negative isolates were

inhibited by AgNPs by sequential interaction with cell wall, metabolism and genetic stability. Gram positive *Staphylococcus aureus* (*S. aureus*) was resistant to AgNPs. The ABC efflux pump system of S. *aureus* was versatile enough to protect the cells.

Conclusion:

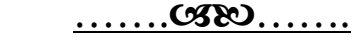
- Estuaries are ecologically sensitive ecosystems and this work is an important contribution to the understanding of how and at what level pollutants such as metals can interfere with the natural environmental variability and influence the abundance and structure of the microbial communities in these environments. It also provides information on the level of metal pollution and identifies locations within the estuary that are likely to serve as reservoirs for tolerant bacteria.
- Trace metals are significant contaminant in the CE due to industrial and agricultural inputs. This study concludes that in the CE, metal concentration encourages the selection and adaptation of different microbial populations. Although, statistically it is demonstrated that metal contamination accounted for a significant amount of variability in the community composition, other possible factors contributing to the determination of the microbial composition have not been quantified in the present study. Controlled contaminant exposure experiments in microcosms are essential to test this hypothesis and remove the background variability present in the estuary. Nevertheless, this study provides significant insights into the bacterial diversity and its response to different levels of metal contamination.

  To the best of my knowledge this is the first report on the metal-microbe interaction in the CE. Anthropogenic-derived metals are implicated as mechanisms maintaining metal resistance in the estuarine environment. This study has also shown the prevalence of multiple metal/ antibiotic resistant bacteria and their differential response towards metal nanoparticles. From this study it can also be postulated that the presence of metal in the environment can maintain the property of antibiotic resistance in the bacteria.

- The knowledge about the mechanisms of microbial resistance to heavy metals will be applicable in environmental biotechnology to mitigate heavy metals from polluted environment in order to restore ecological niches.

Future Line of Work

- Future work based on culture independent bacteria and molecular approaches is needed to address specific mechanisms involved in the association between metal exposure and antibiotic resistance. Quantification of genetic elements that are involved in these mechanisms would yield pertinent information regarding the frequency of antibiotic and metal tolerance genotypes in the CE. A range of novel methods based on RNA and DNA analysis are applied in marine microbiology. Few of these are 16SrDNA for molecular phylogenetic, 454 pyrosequencing, MALDI TOF, DNA microarrays, use of functional probes etc. Microbial rRNA genes extracted from samples can be sequenced and detected directly. These sequences can then be compared with those from other known microorganisms. Additionally, group and taxon-specific oligonucleotide probes can be developed from these sequences making direct visualization of microorganisms in habitats. Another approach is in *situ* activity measurements.
- One potential link between local metal contamination and global climate change phenomena is the inhibition of bacterial nitrous oxide reductase by metal toxicity. This leads to incomplete denitrification and accumulation of the potent greenhouse gas N2O known to interfere with the ozone layer. As excess emission of nitrous oxide leads to climate change, future work will involve the measurement of N_2O concentration along the pollution gradient of CE.
- Metal contamination represents a long-standing, widespread and recalcitrant selection pressure for both environmental and clinical bacteria. Since metal resistance is linked to antibiotic resistance it leads to spread of antibiotic resistant bacteria. Stringent control on use and discharge of metal and antibiotics into the environment should be implemented to avoid the spread of MRB. Spread of MRB can also be controlled by using metal accumulation and bioremediation capacity of microorganisms. Pre-treatment of effluent by consortia of metal resistant bacteria can be exploited to remove, concentrate and recover metals from industrial effluents.



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MEDIA

Readymade Hi-media (Mumbai) was used wherever mentioned. All preparations are in 50% sea water. Composition of the medium are in grams per liter.

Nutrient Agar

Peptone	-	5.0
Beef extract	-	3.0
Sodium chloride	-	5.0
Agar	-	15.0
pH	-	7.3 ± 0.2

Peptone Yeast extract Tryptone *(*PYT 80)

Peptone	-	0.08
Yeast Extract	-	0.08
Triptone	-	0.08
Agar	-	15.0
pH	-	7.3 ± 0.2

Starch Agar

Peptone	-	5.0
Beef extract	-	10.0
Sodium chloride	-	5.0
Soluble starch	-	2.0
Agar	-	15 .0
Ph	-	7.3 ± 0.2

Lipid Hydrolysis Medium

Peptone	-	10
Beef extract	-	10
Sodium chloride	-	5.0
Calcium chloride	-	0.1
Tween	-	1%
Agar	-	12.0
pH	-	7.3 ± 0.2

Gelatin Medium

Peptone	-	10.0
Beef extract	-	10.0
Sodium chloride	-	5.0
Gelatinase	-	4.0
Agar	-	15.0
pH	-	7.3 ± 0.2

Phenolphthalein Phosphate Agar

Peptone	-	5.0
Beef extract	-	3.0
Sodium chloride	-	5.0
Sodium phenolphthalein phosphate	-	0.012
Agar	-	15.0
pH	-	7.3 ± 0.2

DNase Agar W/ Toludine Blue

Tryptose	-	20
Deoxyribonucleic acid	-	2.0
Sodium chloride	-	5.0
Toludine blue	-	0.10
Agar	-	15.0
pH	-	7.3 ± 0.2

Soyabean Casein Digest Agar (SCDA)

Casein enzyme hydrolysate	-	15.0
Peptic digest of Soyabean meal	-	5.0
Sodium chloride	-	5.0
Agar	-	15.0
pH	-	7.3 ± 0.2

Christensen Urea agar base – Urease

Peptone	-	1.0
Dextrose	-	1.0
Sodium chloride	-	5.0
Disodium phosphate	-	1.2
Monopotassium phosphate	-	0.8
Phenol red	-	0.012
Agar	-	15.0
pH	-	7.3 ± 0.2

Luria Bertani Broth

Sodium Chloride	-	10
Tryptone	-	10

Yeast Extract	-	5
pH	-	7.3 ± 0.2

STAINS

- **Acridine Orange**

Acridine orange	-	0.1g
Formalin (5%)	-	100ml

BUFFERS

- **Buffered formalin**

38% Formalin	-	100 ml
Hexamin	-	to saturation

Filter through 0.22µm pore size filter and store at room temperature

SOLUTIONS

- **Antibiotic Cocktail**

Nalidixic acid	-	8mg
Piromedic acid	-	4mg
Pipemedic acid	-	4mg
Saturated NaOH solution	-	50µl
Distilled water	-	10ml

Dissolve the antibiotics separately in saturated sodium hydroxide solution and mix with water. Sterilize by filtration through 0.22µm pore size filter and store in autoclaved vials at 4^0 C in a refrigerator.

REAGENTS

Nitrate

1. Reagent A

N (1-napthyl ethylenediaminedihydrochloride):

0.5g of amine in 500ml distilled water.

2. Reagent B

Sulphanilamide solution:

Dissolve 10g sulphanilamide in a mixture of 100ml conc. HCl and 500 ml distilled water. Make up the volume to 1000ml with distilled water.

3. Reagent C

Conc. Ammonium chloride solution: Dissolve 125g of AR quality ammonium chloride in 500ml of distilled water.

4. Reagent D

Mercuric chloride solution: Prepare 1% mercuric chloride solution by dissolving 1g of HgCl2 in 100ml distilled water.

Phosphate

1. Reagent A

Ammonium molybdate solution:

15g of reagent grade ammonium paramolybdate dissolved in 500mldeionized water.

2. Reagent B

Sulphuric acid solution: (4.5M)

250 ml of Conc. Sulphuric acid added into 750 ml deionized water.

3. Reagent C

Potassium antimonyltartarate solution:

0.34g of potassium antimonyl tartarate in 250ml deionized water.

4. Reagent D

Ascorbic acid solution:

27g ascorbic acid in 500ml deionized water.

5. Mixed reagent:

Mix together 100ml ammonium molybdate solution, 250ml sulphuric acid solution, 50ml potassium antimonyl tartarate.

MIS

- **Reagent 1 - Saponification Reagent**

  Sodium hydroxide (certified ACS) - 45 g

  Methanol (HPLC grade) - 150 ml

  Deionized distilled water - 150 ml

  Combine water and methanol. Add NaOH pellets to the solution while stirring. Stir until the pellets dissolve.

- **Reagent 2 - Methylation Reagent**

  6.00N Hydrochloric Acid - 325 ml

  Methanol (HPLC grade) - 275 ml

  Add acid to methanol while stirring.

- **Reagent 3 - Extraction Solvent**

  Hexane (HPLC Grade) - 200 ml

  Methyl tert-butyl ether (HPLC Grade) - 200 ml

  Add the MTBE to the hexane and stir well.

- **Reagent 4 - Base Wash**

  Sodium hydroxide (certified ACS) - 10.8 g

  Deionized distilled water - 900 ml

Add NaOH pellets to the water while stirring. Stir until the pellets are dissolved.

- **Additional Reagents**

Saturated NaCl: Dissolve 40g ACS NaCl in 100 ml distilled water.

Comet assay

- **Lysis solution:**

0.15M NaCl

10mM TRIS (pH10)

100 mM EDTA

Triton X (1%)

Preparation: 5 ml of lysis solution was prepared by adding150µl of 0.15M NaCl, 50 µl of 10mM TRIS (pH10), 500 µl of 100 mM EDTA. 50µl of 1% Triton X was added fresh before the experiment by stirring for 10 minutes at room temperature. The final volume was made up to 5ml with milli Q water (3750µl).

- **Enzyme digestion solution**

0.15M NaCl

10mM TRIS (pH7.4)

10 mM EDTA

Proteinase K

Preparation : 5 ml of enzyme digestion solution was prepared by adding 150µl NaCl (0.15M), 50µl Tris (pH 7.4,10 mM), 50µl of 10mM EDTA and 250µl of 1mg/ml Proteinase K. The volume was made up to 5ml with milli Q water (4500µl).

- **Eletrophoretic buffer:**

300mM Sodium Acetate

1. 00 mMTris

Preparation: 150 ml of Sodium acetate (300mM) and 50 ml of 100mM Tris was mixed and the pH was adjusted to 9.The final volume was made to 500 ml with distilled water.

- **0.1M ammonium acetate in ethanol:**

  1M ammonium acetate

  Absolute ethanol

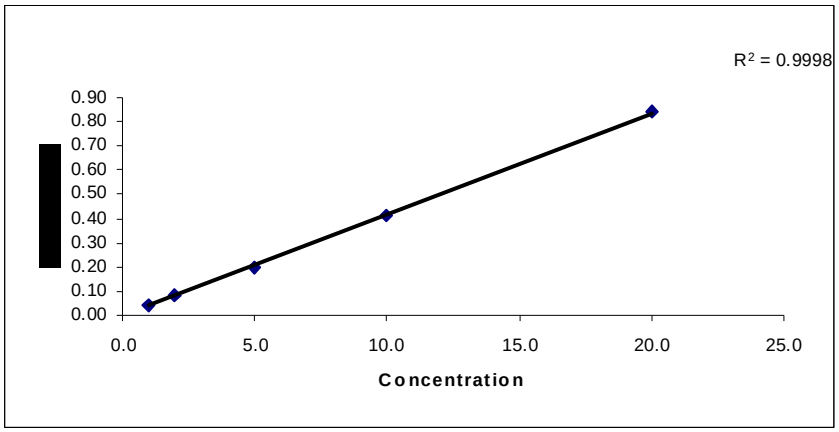
- **SyBr Green**

  1µl of the DNA dye SyBr Green (Sigma Aldrich Cat.No: S9430) was diluted 2000 times with the staining solution buffer. Staining solution buffer contained 50 µl of 10mM Tris and 5µl of 1 mM EDTA. Volume made up to 5 ml with Milli Q water as per the manufacturers' instruction.

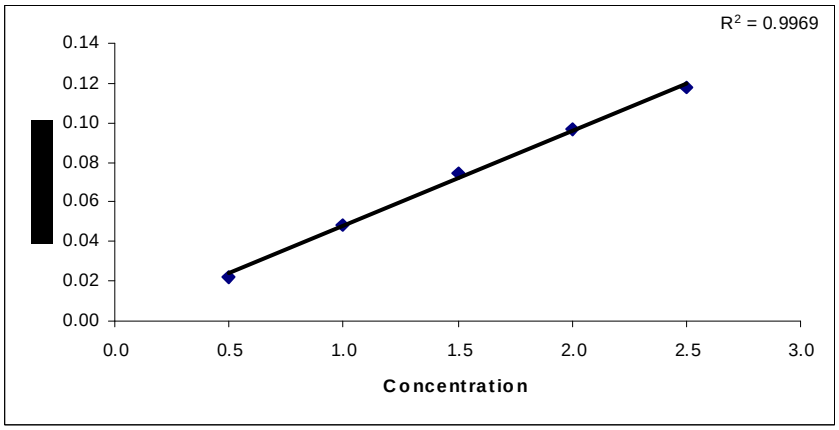
STANDARD GRAPHS

NUTRIENTS

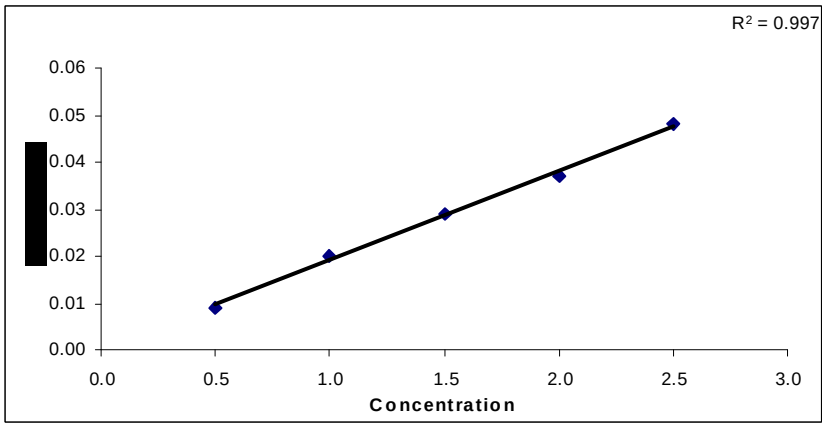
1. Nitrate



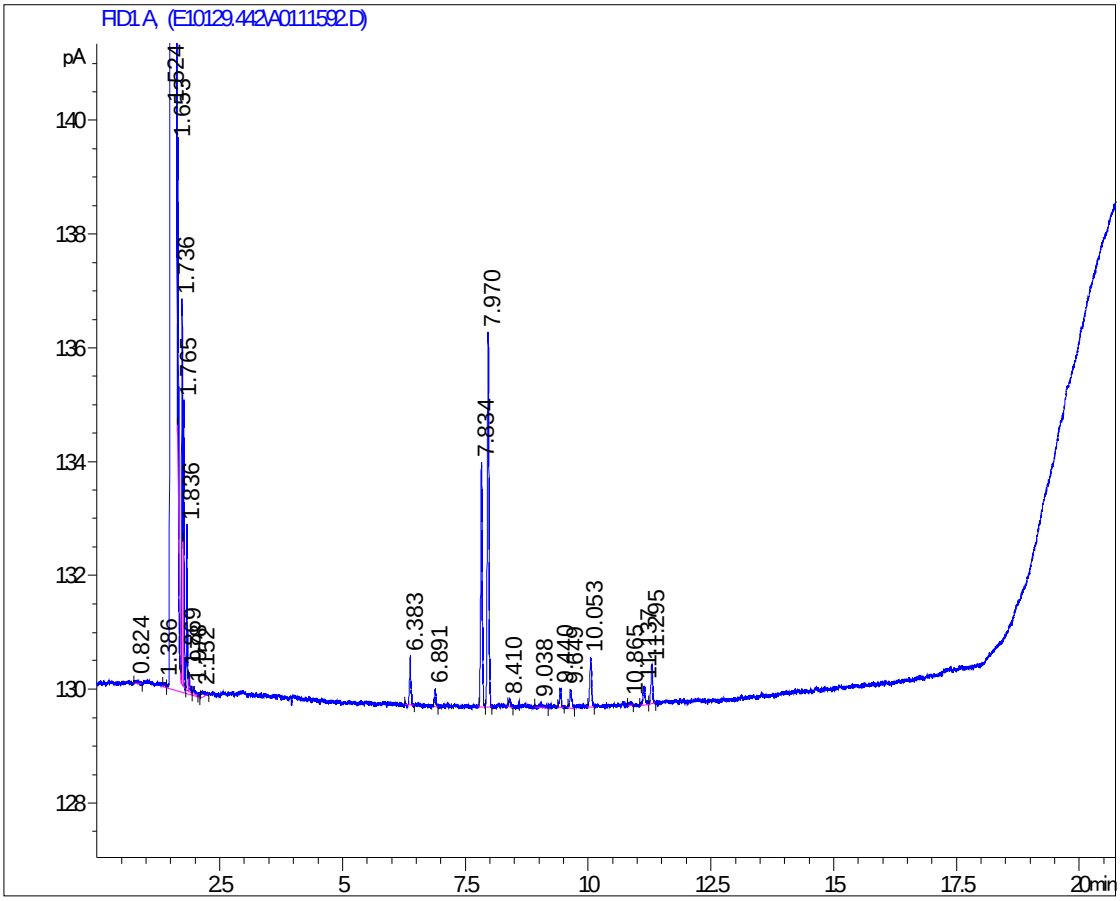
2. Nitrite



3. Phosphate



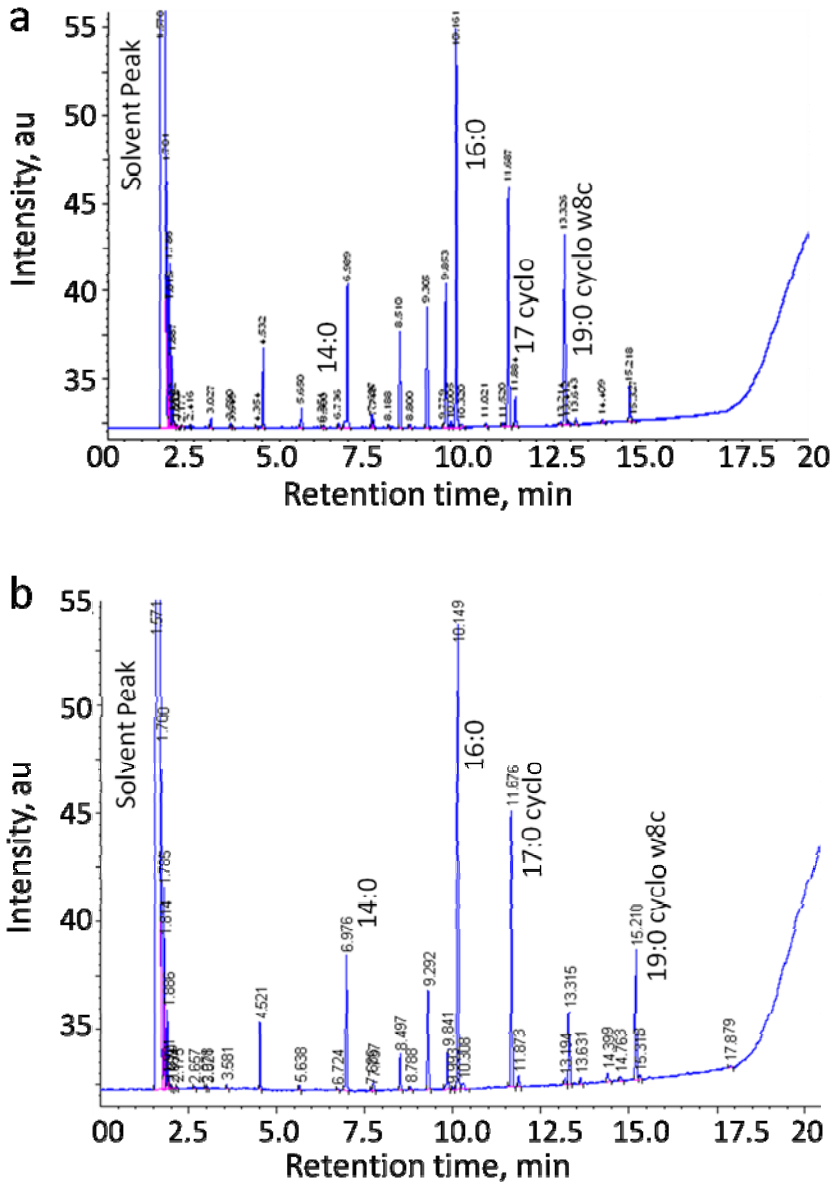
MIS -CHROMATOGRAM AND COMPOSITION - I



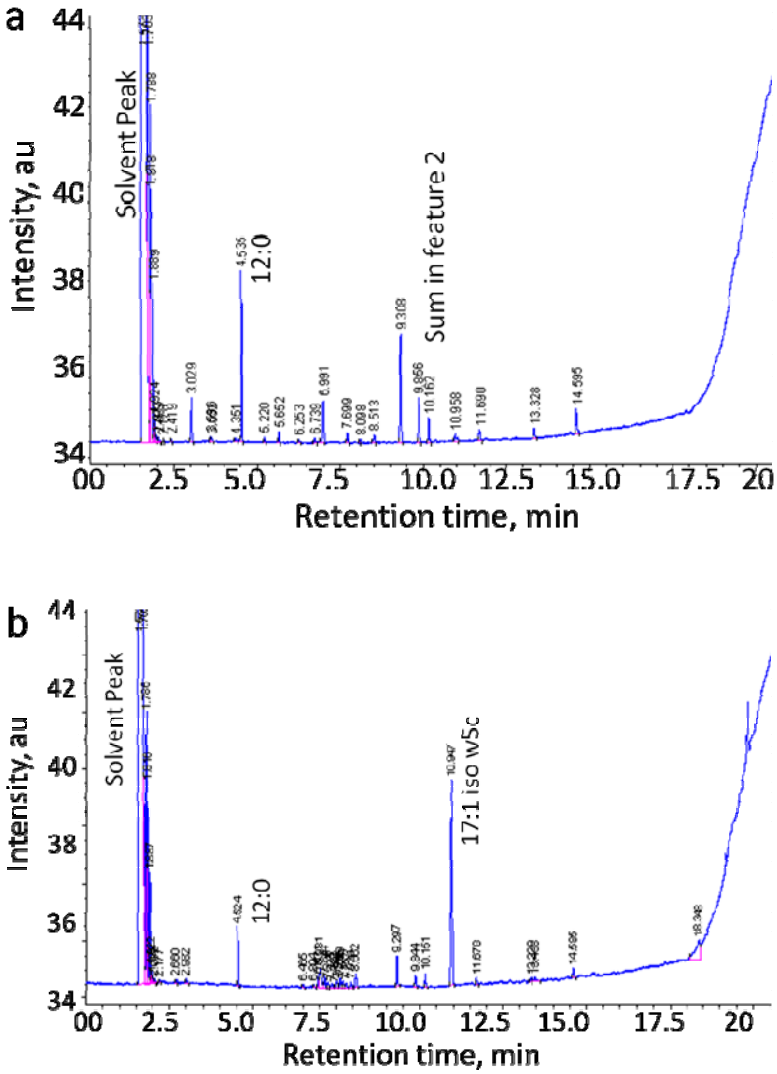
RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.480	584	0.051	----	4.859		----	< min rt	
1.169	114	0.017	----	6.392		----	< min rt	
1.464	3.064E+8	0.023	----	7.048	SOLVENT PEAK	----	< min rt	
1.584	11015	0.015	----	7.317		----	< min rt	
1.664	11202	0.018	----	7.494		----	< min rt	
1.692	9623	0.020	----	7.556		----	< min rt	
1.759	5029	0.022	----	7.707		----	< min rt	
1.839	378	0.021	----	7.883		----	< min rt	
2.664	228	0.023	----	9.720		----		
3.543	-----	---	1.136	11.150	10:0 2OH	----	< min ar/ht	
4.256	162	0.021	1.083	12.001	12:0	0.10	ECL deviates 0.001	Reference -0.002
4.907	740	0.034	1.049	12.613	13:0 iso	0.46	ECL deviates -0.001	Reference -0.005
5.763	398	0.051	----	13.345		----		
5.979	217	0.026	----	13.513		----		
6.115	2008	0.035	1.001	13.619	14:0 iso	1.19	ECL deviates 0.000	Reference -0.004
6.606	1554	0.039	0.986	14.000	14:0	0.91	ECL deviates 0.000	Reference -0.004
7.521	96695	0.035	0.963	14.624	15:0 iso	55.02	ECL deviates 0.001	Reference -0.004
7.652	34740	0.035	0.960	14.713	15:0 anteiso	19.71	ECL deviates 0.000	Reference -0.005
9.082	6079	0.036	0.933	15.627	16:0 iso	3.35	ECL deviates 0.000	Reference -0.005
9.680	7102	0.039	0.924	15.999	16:0	3.88	ECL deviates -0.001	Reference -0.006
9.876	451	0.041	----	16.115		----		
10.125	333	0.030	----	16.263		----		
10.743	18340	0.040	0.911	16.629	17:0 iso	9.87	ECL deviates -0.001	Reference -0.005
10.898	8266	0.043	0.910	16.722	17:0 anteiso	4.44	ECL deviates -0.001	Reference -0.006
11.725	103	0.014	----	17.207		----	< min ar/ht	
11.842	261	0.023	----	17.274		----		
13.100	177	0.019	0.891	18.001	18:0	0.09	ECL deviates 0.001	Reference -0.002
13.901	2107	0.142	0.887	18.464	19:1 iso I	----	> max ar/ht	
14.729	136	0.017	----	18.943		----		
14.836	520	0.044	0.884	19.005	19:0	0.27	ECL deviates 0.005	Reference 0.003
15.603	482	0.045	----	19.452		----		
15.647	341	0.037	----	19.477		----		
15.756	753	0.064	0.881	19.541	18:0 3OH	0.39	ECL deviates -0.009	
16.045	198	0.020	----	19.710		----		
16.538	613	0.048	0.881	19.997	20:0	0.32	ECL deviates -0.003	Reference -0.003
17.913	185	0.022	----	20.797		----	> max rt	
18.376	20551	0.138	----	21.067		----	> max rt	

Library	Sim Index	Entry Name
TSBA6 6.00	0.703	*Bacillus-pumilus*

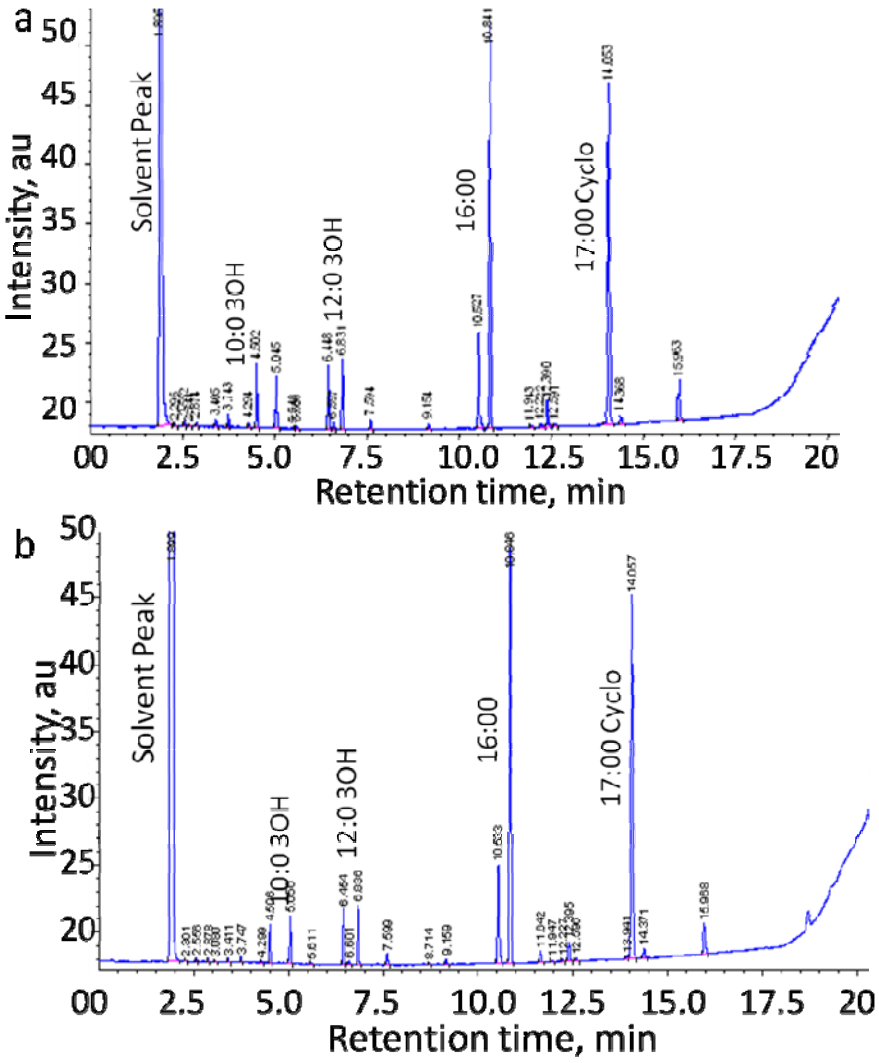
CHROMATOGRAMS OF MDR PATHOGENIC MRB



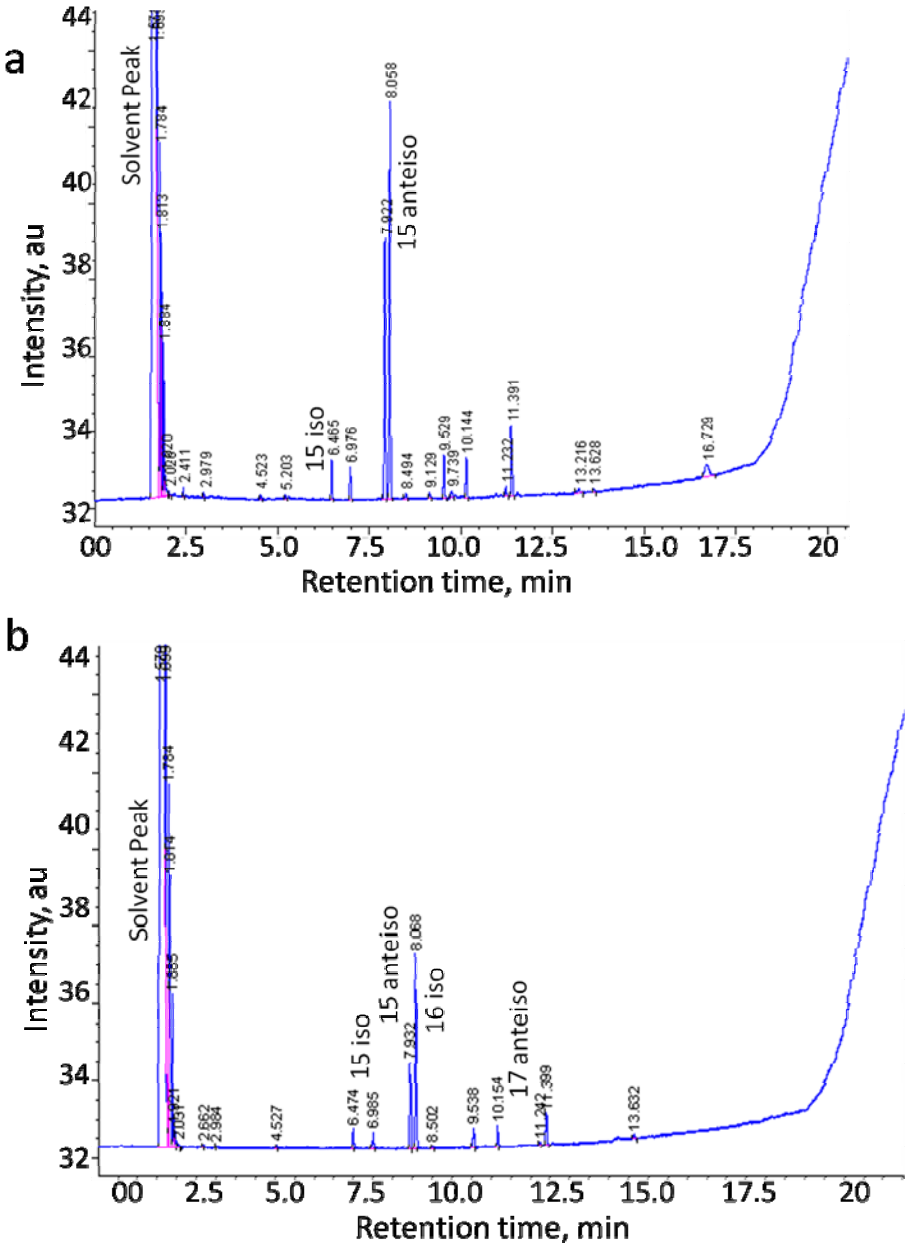
Fatty acid profile of *E. coli* before (a) and after (b) treatment with AgNPs



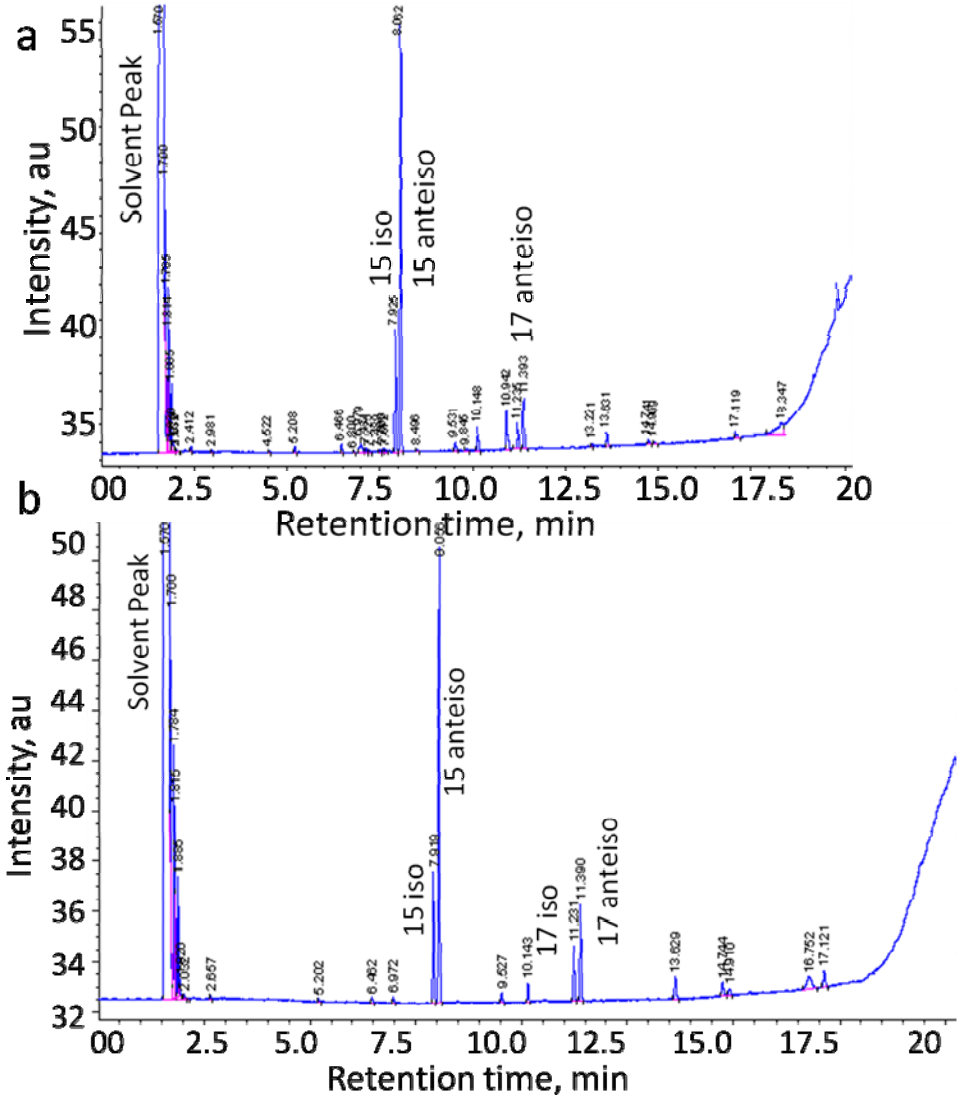
Fatty acid profile of *V.alginolyticus* before (a) and after (b) treatment with AgNPs



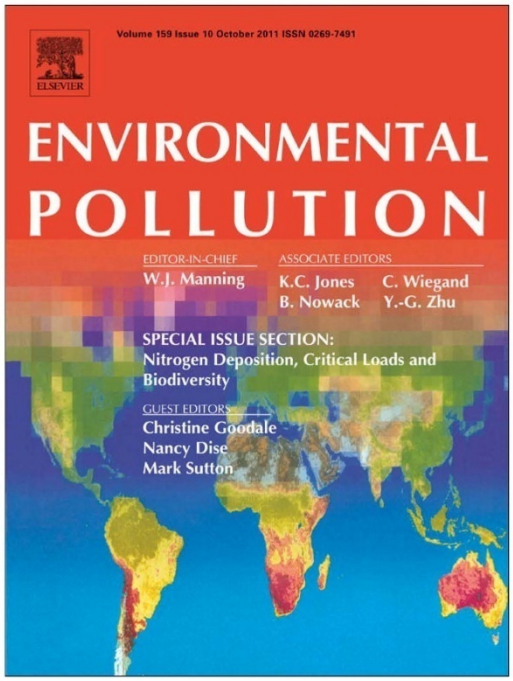
Fatty acid profile of *P.aeruginosa* before (a) and after (b) treatment with AgNPs



Fatty acid profile of *B.* subtilis before (a) and after (b) treatment with AgNPs



Fatty acid profile of *S.aureus* before (a) and after (b) treatment with AgNPs



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Heavy metal pollution exerts reduction/adaptation in the diversity and enzyme expression profile of heterotrophic bacteria in Cochin estuary, India

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ABSTRACT

Over the past three decades heavy metal pollution has increased substantially in Cochin estuary, south west coast of India. Here we studied the distribution, diversity and enzyme expression profile of culturable microbial population along a pollution gradient. The distribution of resistance against 5 mM concentration of Zn, Co, Ni and Cu was observed among 90–100% of bacterial isolates retrieved from highly polluted Eloor, whereas it was less than 40% in Vypin and Munambam. Similarly, there was a difference in the distribution and diversity of bacterial phyla with predominance of Proteobacteria in Eloor and Firmicutes in Munambam and Vypin. We observed that 75–100% of the organisms retrieved from Eloor had low levels of expression for hydrolytic enzyme. In conclusion, the heavy metal pollution in Cochin estuary brought in reduction/adaptation in the distribution, diversity and enzyme expression profile of bacteria, which may impart adverse impacts on ecosystem functioning.

1. Introduction

Microorganisms have been exposed to varying concentrations of heavy metals presumably since the beginning of life (Silver and Phung, 1996; Martinez et al., 2009), and have sustained by maintaining a homeostasis between the available metal concentration and microbial physiology (Hantke, 2001; Kosolapov et al., 2004; Bong et al., 2010). However, in a contaminated environment, the elevated concentrations of heavy metal impinge the conformational structures of nucleic acids and proteins, and consequently form complexes with molecules, which render them inactive (Bong et al., 2010). For example, at millimolar concentrations, Zn ions bind with the cell membrane of bacteria and interfere with cell division (Nies, 1999; Silver and Phung, 2005) in spite of being a micronutrient (Wilson and Reisenauer, 1970).

Effect of heavy metal pollution on the diversity and dynamics of microorganisms in marine environment is a topic of growing environmental concern as it has direct and long lasting impact on ecosystem functioning and are not easily degradable (Valsecchi et al., 1995; Bong et al., 2010). Recently, the anthropogenic contribution of heavy metals to estuarine environment has increased significantly through the discharge of industrial and domestic wastes (Balachandran et al., 2006; Nair et al., 2006; Noah and Oomori, 2006). In general, heavy metal pollution causes unintended alterations in the functioning of marine ecosystems directly or indirectly. The direct impact of heavy metal pollution on microbial ecosystem includes the alterations in the physiology, diversity and abundance of microorganisms, which indirectly affect the biogeochemical cycles and ocean productivity (Haferburg and Kothe, 2007; Chakravarty and Banerjee, 2008; Hoostal et al., 2008; Bong et al., 2010). Hydrolytic enzymes secreted by bacteria are of much importance in marine environment for the processing of polymeric and particulate organic matter to dissolved organic matter and facilitating further passive transportation across the cell membrane of bacteria (Chrost and Rai, 1994; Bong et al., 2010). Heavy metal pollution exerts a selective pressure on microbial community leading to the emergence of resistant strains with apparent reduction in the extracellular enzyme activity of that particular ecosystem (Silver and Misra, 1984; McGrath et al., 2001; Lasat, 2002; Li et al., 2006; Souza et al., 2006; Wang et al., 2007). Such inhibitory effects of Cd, Zn, Ni and Co on expression of bacterial nitrous oxide reductase enzyme were reported, which may lead to the accumulation of potent green house gas N_2O and thus contribute indirectly to global warming (Dickinson and Cecerone, 1986; Minagawa and Zumft, 1988; Sobolev and Begonia, 2008; Haferburg and Kothe, 2010). In addition, the biomagnification of heavy metals may cause chronic and acute ailments in human beings (Förstner and Wittmann, 1979). Therefore, the heavy metal

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pollution and subsequent effect on estuarine microbial diversity and function are of important topics of research.

Like most estuaries, the anthropogenic contribution of heavy metals has increased significantly in Cochin estuary over the past three decades through the discharge of industrial and domestic wastes (Balachandran et al., 2005; Balachandran et al., 2006; Nair et al., 2006). In the present study we assessed the effect of heavy metals, Zn, Cd, Hg, Ni, Cu and Co, on the distribution and diversity of culturable bacteria along a pollution gradient in Cochin estuary. We also investigated the effect of heavy metals on the extracellular enzyme profile of bacterial isolates.

2. Materials and methods

2.1. Description of the study area and sampling

Cochin estuary extends from Thanneermukkam bund in the south to Azhikode at the north (9° 30′–10° 12′N and 76° 10′–76° 29′E). It covers an estimated length of ~60 km and an area of ~21050 ha. It is connected to the Arabian Sea at two locations, Fort Cochin (9° 58′N) and Azhikode (10° 10′N) (Balachandran et al., 2008). And functions as a repository for effluents from more than 240 industries on the banks of river Periyar, the characteristics of which include fertilizer, pesticide, radioactive mineral processing, chemical and allied industries, petroleum refining and heavy metal processing and fish processing (Thyagarajan, 2004). In order to assess the effect of heavy metal on bacterial parameters, three stations lying between the effluent discharge point and barmouth of Cochin estuary were selected. The positions of the sampling stations, Eloor, Vypin and Munambam are shown in Fig. 1. Eloor, designated as a grossly polluted station in this study, is at an intersection where river Periyar carrying industrial effluents join the Cochin estuary. Vypin is situated near the Cochin barmouth and is designated as least polluted station. Munambam is an intermediately polluted station.

Sediment and water samples were collected from each station employing Van Veen grab and 10 L capacity Niskin water sampler respectively. For microbiological analysis, multiple samples of water and sediments were removed aseptically in to sterile polypropylene bottles and maintained at 4 °C until further analysis. Samples for chemical analysis were collected avoiding contamination from all possible sources. Sediment samples were sealed in plastic bags and frozen till analysis. All samples were transported in an ice chest to the laboratory for further analysis.

2.2. Metal analysis of sediment and water samples

One gram of dried and finely powdered sediment samples were digested repeatedly with HF-$HClO_4$-HNO_3, suspended in 0.5 M HCl (25 mL) and analyzed for Zn, Cd, Hg, Ni, Cu and Co using inductively coupled plasma atomic emission spectrometer (ICP-AES) following the standard protocol (Loring and Rantala, 1977). Known volumes of water samples were filtered through pre–weighed Millipore filter paper (0.45 μm) and the filtrate was acidified using concentrated hydrochloric acid. The dissolved metals were extracted using Ammonium Pyrrolidine Dithiocarbamate (APDC) and Methyl Isobutyl Ketone (MIBK) at pH 4.5 and brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 mL with sterile de-ionized water (Smith and Windom, 1972). The extracts were analyzed in the flame for dissolved trace metals.

2.3. Isolation and identification of bacteria

Culturable bacteria present in sediment and water were retrieved on Nutrient agar (Hi-Media laboratories Pvt. Ltd, Mumbai, India) and Peptone Yeast extract and Tryptone medium (PYT80) (Konopka and Zakharova, 1999) following standard microbiology protocols. Nutrient agar is a rich medium which supports the growth of fast growing microorganisms whereas PYT80 medium facilitates the growth of slow growing metal resistant bacteria. A total of 232 morphologically different isolates were purified and preserved in 20% glycerol for further studies. The bacterial isolates were subjected to morphological and biochemical tests viz pigmentation, oxidase, catalase, MRVP, carbohydrate utilization, MOF, antibiotic sensitivity, indole and O/129 sensitivity etc. (Gerhardt et al., 1981) and identified following the scheme of Oliver and Smith (1982). The identification was further confirmed by Microbial Identification System (MIS) operating manual (MIDI, USA). Briefly, the whole cell fatty acids of the bacteria were extracted and methylated according to MIDI protocol and analyzed using gas chromatography system (Agilent GC 6950) and the peaks were compared with the library of Sherlock v6 (MIDI, USA). Previous studies have shown that more than 90% identification of bacteria by fatty acid profile is in accordance with 16S rRNA gene sequencing method at genus level and more than 70% at species level (Osterhout et al., 1991; Tang et al., 1998).

2.4. Screening of metal resistance

Bacterial resistance to heavy metals was examined by the plate diffusion method (Hassen et al., 1998). The glassware were leached in 2 N HNO_3 and rinsed several times with sterile de-ionized water before use to avoid metal contamination. A volume of 500 μl of solution containing a final concentration of 5 mM metal salt ($ZnSO_4{\cdot}7H_2O$, $HgCl_2$, $CoCl_2{\cdot}6H_2O$, $CdCl_2$, $CuSO_4{\cdot}5H_2O$, $NiCl_2$) was poured in to the central well of PYT80 plates and was incubated at 28 ± 2 °C for 24 h to allow diffusion of the metal into the agar. Six strains of bacteria were streaked in a radial fashion on each plate and incubated for seven days at 28 ± 2 °C. All the experiments were done in triplicate and the bacteria which showed visible growth in seven days were counted as metal resistant.

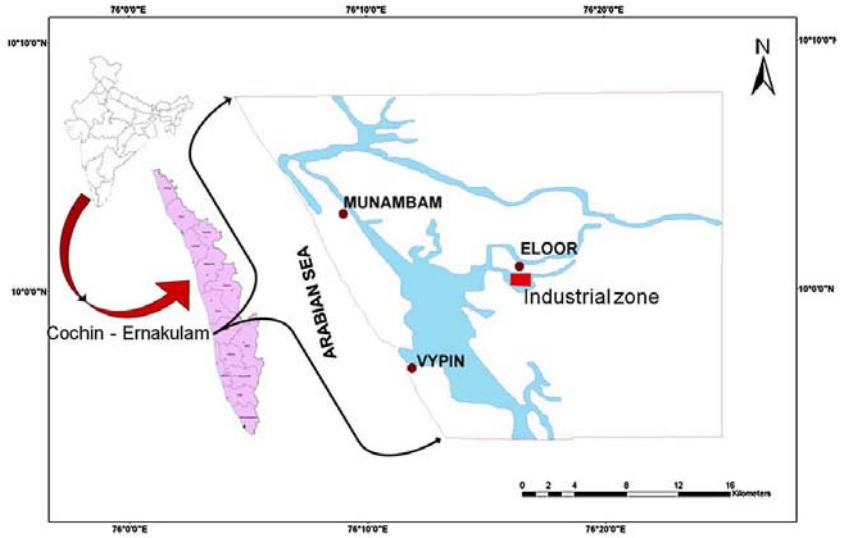

Fig. 1. Map showing study area and station locations.

2.5. Enzyme assays

The isolates were tested for amylase, gelatinase, lipase, and protease following the protocol described by Simbert and Krieg (1981). Isolates were spotted on nutrient agar medium containing different substrates, and the enzyme expression profile was measured as the function of the ability of individual microorganisms to produce clearing zones.

2.6. Statistical analysis

Shannon diversity index (H') was used to assess the diversity and richness of microorganisms (Shannon and Weaver, 1949). Student's *t*-test was used to assess the differences at $p < 0.01$ (Bailey, 1995).

3. Results and discussion

3.1. Heavy metal pollution in Cochin estuary

The concentration of heavy metals in water and sediment samples is presented in Table 1. Here, we observed 2758 mg kg^{-1} Zn in sediment samples collected from Eloor region, which is approximately 2.5 times higher compared to values recorded from previous observations. The heavy metal concentration was higher in all sediment samples than respective water samples. Eloor with its close geographical location to the industrial belt of Cochin receives higher concentrations of heavy metals. The major pollutant in Eloor was Zn with 2758 mg kg^{-1} and 1159 mg L^{-1} in sediment and water respectively. Nickel with 259 mg kg^{-1} and 123 mg L^{-1} respectively, in the sediment and water was the second major pollutant observed in Eloor region. Vypin, which lies outside the reception point of industrial discharge, was found to be the least polluted station with no detectable concentrations of heavy metals except 16 mg kg^{-1} Co in the sediment. The water samples collected from Vypin was relatively unpolluted with all the heavy metals below detectable limits. Munambam was the intermediate region with 290 mg kg^{-1} Zn and 109 mg kg^{-1} Ni as the major pollutants in the sediment. Considering the concentration of all metals, the gradation of pollution at the stations were Vypin < Munambam < Eloor. It is clear that heavy metal pollution has proliferated substantially during the past three decades in Cochin estuary (Balachandran et al., 2005; Nair et al., 2006). During the period of 1976–2000, Zn concentration in sediments of Cochin estuary has increased from 70 to 1266 mg kg^{-1} (Venugopal et al., 1982; Balachandran et al., 2006). If left unchecked, the harmful effects of these heavy metals in the environment would be permanent and irreversible.

3.2. Effect of heavy metal pollution on the distribution and diversity of culturable bacteria

Percentage variation in the distribution of culturable bacteria in water and sediment is given in Table 2. With increase in heavy metal concentration, it was observed that the percentage of Proteobacteria increased irrespective of water or sediment. It was observed that Proteobacteria dominated in Eloor with 78.3 and 88.9% in water and sediment respectively. Among the Proteobacteria thirteen genera were recovered from the study area (Table 3). The high dominance of Proteobacteria may be facilitated by an array of metal transport systems which was reported earlier by genome analysis (Nakagawa et al., 2007). These 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). Increased level of expression of genes encoding efflux proteins has been observed when a *Pseudomonas aeruginosa* without any previous exposure to heavy metals was subjected to

Table 1
Concentration of heavy metal pollutants in the water and sediment samples collected from different sampling locations of Cochin estuary.

Heavy metals	Concentration in water samples (mg L^{-1})			Concentration in sediment samples (mg Kg^{-1})		
	Vypin	Munambam	Eloor	Vypin	Munambam	Eloor
Zn	BDL	80	1159	BDL	290	2758
Cd	BDL	BDL	50	BDL	27	164
Hg	BDL	BDL	BDL	BDL	BDL	BDL
Ni	BDL	56	123	BDL	109	259
Cu	BDL	BDL	97	BDL	42	145
Co	BDL	21	50	16	80	114

BDL – Below detectable limit.

Table 2
Percentage variation in the distribution of culturable bacteria isolated from water and sediment samples of Vypin, Munambam and Eloor.

Phylum	Water			Sediment		
	Vypin	Munambam	Eloor	Vypin	Munambam	Eloor
Proteobacteria	30.0	30.8	78.3	50.0	47.6	88.9
Firmicutes	65.0	61.5	17.4	38.5	38.1	11.1
Actinobacteria	5.0	00	4.3	3.8	9.5	ND
Bacteriodetes	ND	7.7	00	7.7	4.8	ND

ND-Not detected.

Table 3
Diversity of culturable bacteria of water and sediment in the Cochin estuary.

Bacterial species	Water			Sediment		
	Vypin	Munambam	Eloor	Vypin	Munambam	Eloor
Proteobacteria						
Acinetobacter baumannii	–	+	–	+	+	–
Chromobacterium violaceum	+	–	+	–	–	–
Citrobacter koseri	–	–	–	+	–	–
Comamonas acidovorans	–	–	+	+	–	+
Comamonas testosteroni	–	+	+	+	–	+
Kluyvera ascorbata	+	–	–	+	+	–
Listonella anguillarum	+	–	+	+	+	+
Proteus mirabilis	–	–	–	–	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Pseudomonas huttiensis	–	+	+	+	+	+
Serratia marcescens	–	–	+	+	–	+
Shewanella pute faciens	–	–	+	+	–	+
Vibrio cholerae	–	–	+	–	+	+
Vibrio furnissii	+	–	+	–	+	+
Xanthomonas arboricola-pruni	–	–	+	+	–	+
Yersinia pseudo tuberculosis	–	–	+	+	–	+
Firmicutes						
Bacillus cereus	+	–	–	+	–	–
Bacillus filicolonicus	+	+	+	+	–	–
Bacillus marinus	+	+	–	–	+	–
Bacillus mycoides	+	+	–	+	+	–
Bacillus oleronius	+	+	–	–	+	–
Bacillus pumilus	+	+	+	+	+	+
Bacillus sphaericus	+	+	–	+	–	–
Bacillus sp	+	–	–	–	+	–
Exiguobacterium acetylicum	–	–	–	+	–	–
Paenibacillus larvae pulvifaciens	+	–	–	+	–	–
Staphylococcus gallinarum	+	–	+	–	–	+
Actinobacteria						
Cellulomonas turbata	+	–	+	+	+	–
Bacteriodetes						
Myroides odoratus	–	+	–	+	+	–
Shannon H'	2.788	2.458	2.774	3.087	2.692	2.66

heavy metal shock (Teitzel et al., 2006; Martinez et al., 2009). Ivanova et al. observed high resistance of Proteobacteria isolated from different marine sources against heavy metals (Ivanova et al., 2001). In the case of Firmicutes it was observed that the percentage distribution of phyla was higher in water compared to sediment (Table 2). Percentage distribution of Firmicutes was 3–4 times less in Eloor compared to the other two stations. Similarly, diversity of Firmicutes was less in Eloor with *Staphylococcus gallinarum* and *Bacillus pumilus* as the only isolates retrieved, whereas 10 species were retrieved from water sample of Vypin. Shannon index showed decrease from 3.09 to 2.66 between sediment samples of Vypin and Eloor ($p < 0.01$), which indicates that the heavy metal had influenced the richness and evenness of bacterial diversity.

3.3. Effect of metal pollution on enzyme expression profile

Heavy metal pollution is a major environmental concern which may influence the bacterial activity and productivity in a system through inhibiting the expression of hydrolytic enzymes (Dell' Anno et al., 2003). Fig. 2 shows the histogram of hydrolytic enzyme expression in bacterial isolates from water and sediment samples. The enzyme expression profile was measured as the function of the ability of individual microorganisms to produce clearing zones in substrate enriched solid media. Microorganisms are classified based on the diameter of clearing zones into low (0–10 mm diameter), medium (10–20 mm diameter) and high (>20 mm diameter) expression classes. 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. It is significant to note that ~40% of the organisms isolated from sediment of Vypin had high protease enzyme expression profile and 25–35% of isolates have high expression profile for amylase, lipase and gelatinase enzymes. This variability in enzyme expression profile may be either due to suppression of the expression of certain enzyme of same bacteria in different stations or the heavy metal actually selected different bacteria with different enzyme expression profile. It was interesting to note that same species of bacteria isolated from pollution gradient has different enzyme expression profile (Supplementary data Table S1). *P. aeruginosa* and *B. pumilus* were selected as candidate strains considering their presence in all the study areas. *P. aeruginosa* and *B. pumilus* isolated from sediment samples of Vypin produced a protease activity of 22 mm diameter each, whereas the same organisms isolated from Eloor could produce only 8 and 6 mm diameter clearing zone respectively. It is known that enzymatic hydrolysis of large dissolved and particulate organic matter to micromolecules of less than 600 Da is the vital process in sustaining primary productivity in marine environment (Weiss

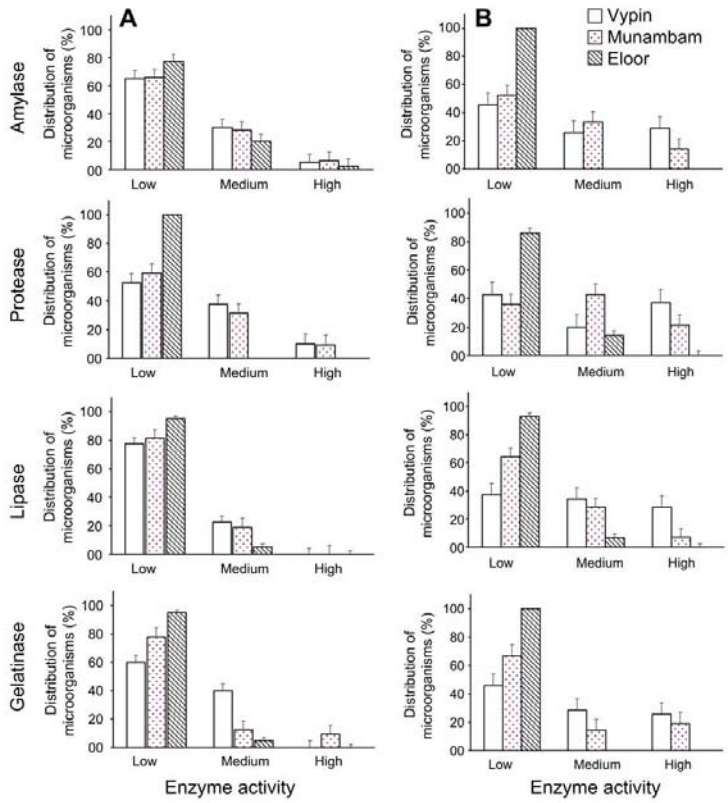

Fig. 2. Histogram showing the percentage distribution of hydrolytic enzyme expression profile (±SD) of culturable bacteria isolated from (A) water and (B) sediment. Low represents 0–10 mm, medium 10–20 mm and high >20 mm of clearing zone in media.

et al., 1991; Hoppe et al., 2002; Yamada and Suzumura, 2010). Therefore, we assume that the reduced enzyme expression profile of microorganisms in Eloor may adversely influence the hydrolysis of large organic matter and may further results in poor cycling of organic nutrients. However, further studies integrating the molecular and biochemical tools are required to explain the correlation between heavy metal induced repression of microbial enzyme profile and productivity in Cochin estuary.

3.4. Bacterial resistance to metals

Fig. 3 shows the percentage of bacterial isolates resistant to heavy metals Zn, Cd, Hg, Ni, Cu and Co. A significant population of the bacterial isolates retrieved from sediment and water samples of Eloor region exhibited resistance against all the metals used in the present study. Microbial communities have been used to reveal the long-term consequences of heavy metal contamination. A direct relationship was observed between environmental metal concentration and microbial community tolerance (Diaz-Ravina et al., 1994; Pennanen et al., 1996; Lock and Janssen, 2005). Thus metal resistance among the bacterial isolates is a direct indication of the exposure of microbial population to heavy metals. It is observed that 90–100% of the bacteria retrieved from Eloor region are resistant to 5 mM concentration of Zn, Co, Ni and Cu, 50–60% to Cd and 20–30% to Hg. The resistant population was restricted to below 20% in the sediment and water of less polluted Vypin and less than 30% in Munambam with an intermediate status of pollution. Mercury resistance was not observed in bacterial isolates from both Vypin and Munambam. Number of isolates resistant to metals was high in the sediment compared to water. This increase might be due to the continuous accumulation of heavy metals in the sediment. It is evident from this study that the heavy metal pollution in Eloor region might have propagated the evolution of a microbial population highly resistant to heavy metals. It is interesting to note that 20–30% of the microbes retrieved from both sediment and water samples of Eloor exhibited resistance to Hg, a heavy metal which was not detected in any of the samples analyzed. This may be due to the ability of the same microorganism to be resistant to one or a group of heavy metals (Silver and Phung, 1996; Barkay et al., 2003; De et al., 2003) or the natural flora is adapted to Hg resistance (Ramaiah and De, 2003). It was demonstrated earlier that the efflux pumps of gram negative bacteria are not specified for a particular compound, rather 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).

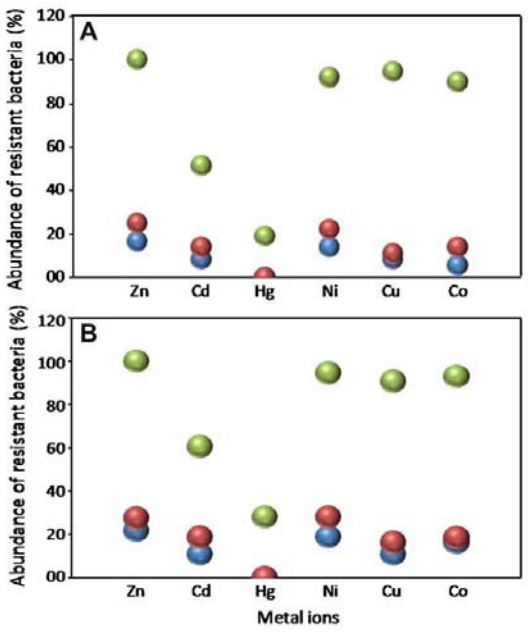

Fig. 3. Percentage of metal resistant bacteria isolated from water (A) and sediment (B) samples of Vypin (blue), Munambam (red) and Eloor (Green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The present study concludes that in Cochin estuary the heavy metal accumulation has resulted in reduction/adaptation of bacterial distribution, diversity and enzyme expression profile and this is proportionate to the extent of pollution. Therefore, the heavy metal pollution and emergence of resistance strains in Cochin estuary is an environmental problem which demands immediate attention as it could have long-term influence on estuarine as well as human health.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.envpol.2011.05.009.

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ORIGINAL ARTICLE

Sequential interactions of silver–silica nanocomposite (Ag–SiO_2NC) with cell wall, metabolism and genetic stability of *Pseudomonas aeruginosa*, a multiple antibiotic-resistant bacterium

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Significance and Impact of the Study: Although the synthesis, structural characteristics and biofunction of silver nanoparticles are well understood, their application in antimicrobial therapy is still at its infancy as only a small number of microorganisms are tested to be sensitive to nanoparticles. A thorough knowledge of the mode of interaction of nanoparticles with bacteria at subcellular level is mandatory for any clinical application. The present study deals with the interactions of Ag–SiO_2NC with the cell wall integrity, metabolism and genetic stability of *Pseudomonas aeruginosa*, which would contribute substantially in strengthening the therapeutic applications of silver nanoparticles.

Keywords
antibacterial, comet assay, *Pseudomonas aeruginosa*, silver nanoparticles, viable but nonculturable.

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Abstract

The study was carried out to understand the effect of silver–silica nanocomposite (Ag–SiO_2NC) on the cell wall integrity, metabolism and genetic stability of *Pseudomonas aeruginosa*, a multiple drug-resistant bacterium. Bacterial sensitivity towards antibiotics and Ag–SiO_2NC was studied using standard disc diffusion and death rate assay, respectively. The effect of Ag–SiO_2NC on cell wall integrity was monitored using SDS assay and fatty acid profile analysis, while the effect on metabolism and genetic stability was assayed microscopically, using CTC viability staining and comet assay, respectively. *Pseudomonas aeruginosa* was found to be resistant to β-lactamase, glycopeptidase, sulfonamide, quinolones, nitrofurantoin and macrolides classes of antibiotics. Complete mortality of the bacterium was achieved with 80 μg ml^{-1} concentration of Ag–SiO_2NC. The cell wall integrity reduced with increasing time and reached a plateau of 70% in 110 min. Changes were also noticed in the proportion of fatty acids after the treatment. Inside the cytoplasm, a complete inhibition of electron transport system was achieved with 100 μg ml^{-1} Ag–SiO_2NC, followed by DNA breakage. The study thus demonstrates that Ag–SiO_2NC invades the cytoplasm of the multiple drug-resistant *P. aeruginosa* by impinging upon the cell wall integrity and kills the cells by interfering with electron transport chain and the genetic stability.

Introduction

Emergence of multiple antibiotic-resistant pathogenic bacteria due to the indiscriminate use of antibiotics has necessitated the search for alternate strategies for controlling these pathogens. Antimicrobial properties of silver nanoparticles (AgNPs) have been demonstrated against a wide range of microorganisms (Sondi and Salopek-Sondi 2004; Panacek *et al.* 2006; Kim *et al.* 2007; Pal *et al.* 2007; Srivastava *et al.* 2007; Choi and Hu 2008; Lut *et al.* 2008; Ayala-Nunez *et al.* 2009; Rai *et al.* 2009; Kalishwaralal *et al.* 2010; Lara *et al.* 2010; Li *et al.* 2010; Mandal

et al. 2011; Beer *et al.* 2012), and no documented report on the emergence of bacterial resistance to silver nanoparticles is available. However, it is not clear whether AgNPs function similar to silver ions or differently. Silver ions damage the membrane proteins, interrupt electron cycle and induce DNA dimerization through the interaction with thiol (S-H) groups of amino acids and other compounds (Feng *et al.* 2000; Gunawan *et al.* 2011). It was proposed that AgNPs upon contact with the cell wall of *Escherichia coli* initiate a chain of reactions, including the expression of a number of envelop proteins (OmpA, OmpC, OmpF, OppA and MetQ), resulting in the formation of permeable pits (Vara 1992; Lok *et al.* 2006). A major prerequisite for achieving maximal antimicrobial activity of AgNPs is to maintain their sensitive surface chemistry, colloidal stability and nanosize (Jana *et al.* 2007). However, in many cases, the metal nanoparticles showed aggregation in aqueous solutions, resulting in retarded biological activity (Jana *et al.* 2007). Although several strategies have been reported for producing biofunctional AgNPs with enhanced water solubility and monodispersity, silica coating is the widely accepted one (Jana *et al.* 2007; Thomas *et al.* 2008).

In this study, we investigated the effect of silver–silica nanocomposite (Ag–SiO_2NC) on the cell wall integrity, metabolism and genetic stability of a multiple antibiotic-resistant strain of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is an aerobic, nonfermenting Gram-negative rod bacterium that is important in the aetiology of nosocomial infections. This bacterium is notorious for its ability to colonize various sites of the human body, including sputum, cornea, nasal mucosa and wet skin, causing a range of diseases, from simple skin infection to fulminant septicaemia (Lee *et al.* 2012). Being a nosocomial pathogen, it is possible that *P. aeruginosa* may be co-evolving with the antibiotics, thus requiring more efficient strategies for controlling the infections. Coating the surgical devices with AgNPs has been proposed as one of the efficient methods for preventing the transmission of this pathogen (Furno *et al.* 2004). Here, we report how Ag–SiO_2NC impinges upon the cell wall integrity and interferes with electron transport chain and genetic stability of a multiple antibiotic-resistant strain of *P. aeruginosa*.

Results and discussion

Continuous evolution of antibiotic-resistant mechanisms among nosocomial pathogens demands the identification of alternate strategies for countering their threat to both humans and animals. The experimental strain of *P. aeruginosa* showed resistance to all the cell wall (β-lactamase and glycopeptidase classes) and nucleic acid synthesis (sulfonamide, quinolones and nitrofurantoin classes)-inhibiting antibiotics used in the study (Table S1). It was also resistant to the Macrolid class protein synthesis inhibitor, azithromycin, but was sensitive to the aminoglycosides (amikacin and gentamycin), tetracyclines (tetracyclin and oxytetracyclin) and chloramphenicol class of protein synthesis-inhibiting antibiotics. Recent reports on co-evolution of antibiotic resistance among microorganisms reiterate the importance of silver compounds with AgNPs as a promising substitute (Rai *et al.* 2012). Compared to silver compounds, Ag–SiO_2NC has better antibacterial properties owing to their large surface area, positive surface charge and monodispersity. Net positive charge of SiO_2 facilitates more number of nanoparticles to interact with negatively charged surface of bacteria, resulting in highly efficient antimicrobial activity (Jana *et al.* 2007). Interestingly, we observed that the death rate of *P. aeruginosa* increased with increasing concentration of Ag–SiO_2NC (13·5% on exposure to 20 $\mu g\ ml^{-1}$) and attained 100% mortality with 80 $\mu g\ ml^{-1}$ (Fig. 1).

Although the antimicrobial mechanisms of silver compounds are well documented, the exact mode of action of AgNPs remains unclear (Gunawan *et al.* 2011; Rai *et al.* 2012). Here, we observed sequential mode of interaction of Ag–SiO_2NC with the test bacterium at subcellular level. Continuous exposure of *P. aeruginosa* to Ag–SiO_2NC for 110 min reduced the cell wall integrity to 70% (Fig. 2). Cell wall integrity is expressed as a function of its sensitivity towards detergent-mediated cell lysis (Lok *et al.* 2006). Cell wall with damaged proteins will be highly prone to detergents, showing sharp decline in the optical density (OD_{600}) due to the leakage of cell content. Sondi and Salopek-Sondi (2004) observed the localization of AgNPs on the surface of *E. coli*, while particles below 10 nm size penetrated the cell membrane. Cell membrane is the first site of action of AgNPs, where they initiate a cascade of reactions, including

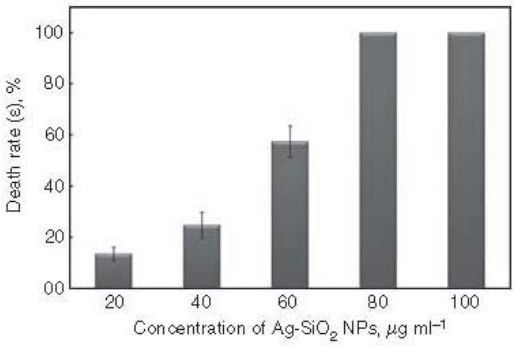

Figure 1 Responses of *Pseudomonas aeruginosa* towards increasing concentrations of Ag SiO_2NC. Results are expressed as death rate ± SD.

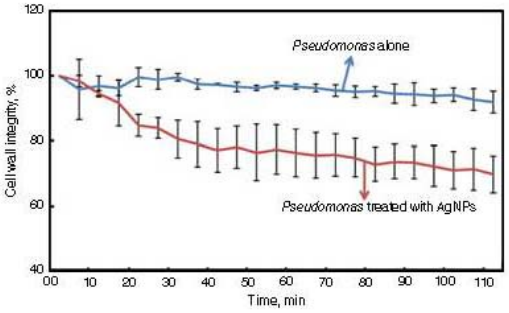

Figure 2 Cell wall integrity of *Pseudomonas aeruginosa* on exposure to Ag SiO_2NC for different time intervals. Results are expressed as cell wall integrity (%) ±SD.

the expression of a number of envelope proteins (Lok *et al.* 2006) and disruption of barrier compounds (Vara 1992; Lok *et al.* 2006). We also observed major changes in the proportion of fatty acids such as 3OH-10:0, 2OH-12:0, 3OH-12:0, 16:0, Cydo-17:0, Cyclo w8c – 19:0, w7c-18:1 and w6c-18 in *P. aeruginosa* after treating with Ag–SiO_2NC, but no significant changes in their profiles were seen (Table 1). Bacteria maintain homoeostasis between the external environment and cell physiology by adjusting the membrane fluidity via altering the proportions of fatty acids. However, severe alterations may impinge upon the cell wall integrity, resulting in the dissipation of transmembrane gradients of protons and ions (Mrozik *et al.* 2004). In the present case, we presume that the alteration in the proportion of fatty acids may be responsible for the translocation of Ag–SiO_2NC in to the cytoplasm.

Table 1 Comparison of the fatty acid composition of *Pseudomonas aeruginosa* before and after treatment with Ag SiO_2NC

	Proportion of fatty acid (%)	
Fatty acid	Before treatment	After treatment
10:0 3OH	4·42	2·66
12:00	3·96	3·62
11:0 3OH	0·1	ND
12:0 2OH	4·89	4·35
12:1 3OH	0·53	0·27
12:0 3OH	5·47	4·71
14:00	0·8	0·85
15:0 anteiso	ND	0·1
16:00	33·91	35·08
17:1 iso w5c	ND	0·97
17:0 iso	0·11	0·13
17:1 w8c	0·2	0·18
17:0 cyclo	2·21	1·68
17:00	0·22	0·24
18:00	0·74	0·81
19:0 cyclo w8c	3·68	2·84
Summed feature 3*	8·58	8·66
Summed feature 8*	30·17	32·84

Results are presented as percentage of the total fatty acids.
*Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature three contains 16:1 w7c/16:1 w6c; Summed feature eight contains 18:1 w7c and 18:1 w6c.

The bacterial metabolism was measured as the ability of electron transport chain of metabolically active cells to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) into red fluorescent formazan. A gradual decrease in the metabolically active cells against increasing concentrations of Ag –SiO_2NC was recorded (Fig. 3). When all the cells are metabolically active, the ratio of metabolically active/total cells will be close to 1. High ratio of 0·8 was observed in the control (without Ag–SiO_2NC), whereas in the presence of Ag–SiO_2NC, the ratio decreased close to zero (100 $\mu g\ ml^{-1}$). Although metabolically active, the cultivability of the bacteria was completely lost at 80 $\mu g\ ml^{-1}$ concentration of Ag–SiO_2NC. This discrepancy could be attributed to cells being viable but non-culturable (VBNC) due the bacteriostatic activity of Ag–SiO_2NC as was observed in the difference in bacterial counts between CTC and death rate assay. Evidently, many bacteria are capable of entering into VBNC state to protect themselves from natural stresses (Oliver 2005; Griffitt *et al.* 2011).

It was observed that DNA of 68% of cells was damaged after 1-h exposure to Ag–SiO_2NC, whereas DNA of all the cells in untreated group remained intact (Fig. 4). This can be attributed to the reactive oxygen species (ROS) generated through the release of Ag^+ ions by Ag–SiO_2NC on contact with cytoplasmic proteins (AshaRani *et al.* 2009). ICP-MS analysis recorded 0·136 and 0·126 ppm of available silver ions from 80 and 100 $\mu g\ ml^{-1}$ concentrations of Ag–SiO_2NC, respectively (Fig. S1). ROS are a group of highly reactive species formed via the activation/reduction of molecular oxygen and includes singlet oxygen ($^1O_2$),

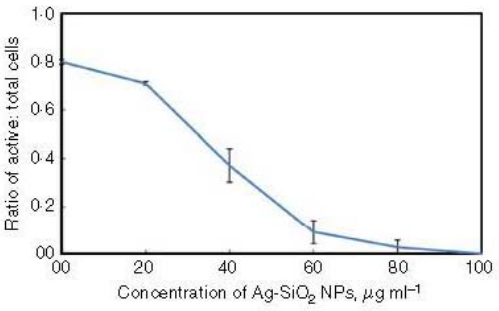

Figure 3 Effect of Ag SiO_2NC on metabolic activity of *Pseudomonas aeruginosa*. Results are expressed as ratio of active/total cells observed in dead/live staining.

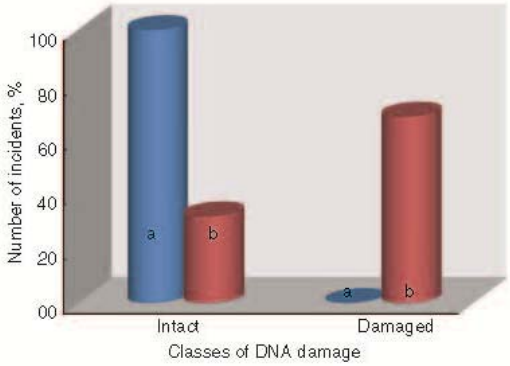

Figure 4 Comet assay histogram of *Pseudomonas aeruginosa* (a) before and (b) after treatment with Ag SiO_2NC. Comets are classified based on tail length into intact (0 10 μm) and damaged (>10 μm) groups.

superoxide anion (O_2^{-*}), hydroxyl radical (HO*) and hydrogen peroxide (H_2O_2). Among the different propositions describing the antibacterial properties of AgNPs, the release of silver ions and subsequent induction of ROS are the prominent ones (Choi *et al.* 2008; Navarro *et al.* 2008; Wigginton *et al.* 2010; Badawy *et al.* 2011). Although minimum concentrations of ROS are necessary for maintaining the regular metabolism of cells, elevated levels can induce DNA damage and breakage (Anas *et al.* 2008). Short lifespan (250 ns) and narrow diffusion power (45 nm) of ROS (Asok *et al.* 2012) demand AgSiO_2NC placed in the proximity of DNA to attain a substantial damage. Silver ions form strong adducts with purine and pyrimidine bases of DNA (Wang *et al.* 2010), and therefore, the substantial DNA damage observed here is a possibility. Ag–SiO_2NC - induced DNA breakage in *P. aeruginosa* was confirmed in the comet assay. In conclusion, the study proposes that Ag –SiO_2NC is a potential antibacterial agent, and follows sequential interaction with the cell wall, metabolism and genetic stability of the multiple antibiotic-resistant *P. aeruginosa*. Ag–SiO_2NC intrudes the cytoplasm by inducing damages to the cell wall and kills the cells by interfering with electron transport chain and genetic stability. An extension of this work with species-specific marker conjugated Ag–SiO_2NC, and targeting of specific pathogens is proposed, which will have considerable positive impact on antimicrobial therapy.

Experimental Procedures

Bacteria and nanoparticles

Pseudomonas aeruginosa strain was obtained from the Marine Microbial Reference Facility (MMRF) of CSIR – National Institute of Oceanography – Regional Centre Cochin India. *Pseudomonas aeruginosa* was inoculated into Luria Bertani (LB) media and grown overnight at 28 ± 2°C on a shaker at 120 rev min^{-1}. Working cultures of *P. aeruginosa* were maintained on LB agar slants and subcultured every 2–3 weeks. The purity of the culture was confirmed by the fatty acid profile.

Ag–SiO_2NC was prepared using Ag–SiO_2 sol and employing tetraethyl orthosilicate-(TEOS), ethanol, distilled water and silver nitrate as precursors. The detailed procedure of nanoparticles synthesis and its characterization using X-ray powder diffractometer and Transmission Electron Microscopy techniques are discussed elsewhere (Thomas *et al.* 2008). Histogram of the particle sizes obtained from transmission electron microscope showed that the mean particle size was 5–6 nm. The available Ag in Ag–SiO_2NC phosphate-buffered saline (PBS) was measured using inductively coupled plasma mass spectrometry (ICP-MS).

Bacterial sensitivity towards antibiotics and Ag–SiO_2NC

Sensitivity of *P. aeruginosa* to twelve antibiotics was tested following standard disc diffusion technique. LB agar plates were swabbed with overnight-grown *P. aeruginosa* suspension cultures, and commercially available antibiotic discs (HiMedia, Bangalore, India) were placed on the agar and incubated at 28 ± 2°C for 24 h. The zone of inhibition was recorded at the end of the incubation period. The antibiotics tested were ampicillin 25 μg, azithromycin 15 μg, amikacin 10 μg, chloramphenicol 30 μg, ciprofloxacin 10 μg, gentamycin 30 μg, vancomycin 10 μg, nalidixic acid 30 μg, nitrofurantoin 100 μg, oxytetracycline 30 μg, trimethoprim 10 μg and tetracycline 10 μg.

Sensitivity of *P. aeruginosa* against Ag–SiO_2NC was tested following standard death rate assay (Asok *et al.* 2012). Briefly, the overnight-grown *P. aeruginosa* cells (10^6 cells ml^{-1}) were mixed with different concentrations of Ag–SiO_2NC (0, 20, 40, 60, 80 and 100 μg ml^{-1}) and incubated at room temperature for 1 h. Subsequently, 100-μl aliquots were plated in triplicate over the surface of a nutrient agar plate and incubated at 28 ± 2°C for 24 h before enumerating total number of colonies. Death rate 'ε' was calculated, using the following equation:

$$\text{Death rate}(\varepsilon)\% = \frac{(N_1 \quad N_2)}{N_2} \times 100,$$

where N_1 and N_2 are the number of colonies grown on the control and experimental plate, respectively.

Also, the number of metabolically active and dead cells in the experimental groups was counted microscopically after staining with BacLight Redoxsensor CTC viability kit

(Molecular Probes; Invitrogen, Eugene, OR, USA), following the protocols suggested in the product brochure.

Effect of Ag–SiO_2NC on cell wall integrity and genetic stability of *Pseudomonas aeruginosa*

The effect of Ag–SiO_2NC on cell wall integrity and genetic stability of *P. aeruginosa* was investigated by SDS assay (Lok *et al.* 2006) and comet assay (Singh *et al.* 1999), respectively. For SDS assay, *P. aeruginosa* cells from an overnight culture were washed copiously with sterile PBS and dispensed in the wells of a sterile microplate to a volume of 200 μl. After measuring the initial absorbance, the test solution was supplemented with Ag–SiO_2NC and SDS (0·1%). Control wells without Ag–SiO_2NC also were maintained. The absorbance at 600 nm was recorded every 15 min for a period of 2 h. The decrease in absorbance compared to the initial reading was plotted against time. All the experiments were carried out three times in duplicate. The fatty acid profile of *P. aeruginosa* before and after treatment with Ag–SiO_2NC was analysed. The log phase cells of *P. aeruginosa* were treated with Ag–SiO_2NC for 3 h, and whole cell fatty acids were extracted, methylated according to standard protocols and analysed using a gas chromatography system 16890N Network GC system, Agilent Technologies Inc., Wilmington, DE).

For comet assay, 10^6 bacterial cells before and after Ag–SiO_2NC treatment were mixed with 100 μl of 0·5% low-melting-point agarose prepared in TAE buffer containing RNAse (5 g ml^{-1}), SDS (0·25%) and lysozyme (0·5 mg ml^{-1}). Bacterial cells impregnated in agarose solution were spread over a microscopic slide precoated with thin layer of agarose (0·5%). The cells on the slides were lysed at 37°C for 1 h, by immersing in a lysis solution, followed by incubation in an enzyme solution for 2 h at 37°C. Subsequently, the slides were equilibrated with 300 mmol l^{-1} sodium acetate and subjected for electrophoresis at 25 V for 1 h. Following electrophoresis, the slides were immersed in 1 mol l^{-1} ammonium acetate in ethanol for 30 min, and then in absolute ethanol for 1 h, air-dried at 25°C, immersed in 70% ethanol for 30 min and air-dried. The slides were stained with SYBR green and comets were observed under a fluorescent microscope. The nucleic acids were classified based on comet length as intact (0–10 μm) and damaged (>10 μm) groups and expressed as % of incidence in the histogram. The assay was repeated three times in duplicates.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Antibiogram of *Pseudomonas aeruginosa* (R, resistant; S, sensitive)

Figure S1 Silver ions released from Ag–SiO_2NC.

Coral Reefs
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REPORT

UV-absorbing bacteria in coral mucus and their response to simulated temperature elevations

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Abstract Reef-building corals encompass various strategies to defend against harmful ultraviolet (UV) radiation. Coral mucus contains UV-absorbing compounds and has rich prokaryotic diversity associated with it. In this study, we isolated and characterized the UV-absorbing bacteria from the mucus of the corals *Porites lutea* and *Acropora hyacinthus* during the pre-summer and summer seasons. A total of 17 UV-absorbing bacteria were isolated and sequenced. The UV-absorbing bacteria showed UV absorption at wavelengths ranging from $\lambda_{max} = 333$ nm to $\lambda_{min} = 208$ nm. Analysis of the DNA sequences revealed that the majority of the UV-absorbing bacteria belonged to the family *Firmicutes* and the remaining belonged to the family *Proteobacteria* (class *Gammaproteobacteria*). Comparison of the sequences with the curated database yielded four distinct bacterial groups belonging to the genus *Bacillus, Staphylococcus, Salinicoccus* and *Vibrio*. The absorption peaks for the UV-absorbing bacteria shifted to the UV-A range (320–400 nm) when they were incubated at higher temperatures. Deciphering the complex relationship between corals and their associated bacteria will help us to understand their adaptive strategies to various stresses.

Keywords *Porites lutea* · *Acropora hyacinthus* · UV-absorbing bacteria · Coral · *Firmicutes*

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Introduction

Coral reefs are unparalleled in terms of both prokaryotic and eukaryotic diversity. Bacteria associated with corals occupy different niches including the surface mucus layer (Ritchie and Smith 2004), tissues (Ceh et al. 2011) and skeleton (Sweet et al. 2011a). The immense diversity of bacteria associated with different species of corals was reported (Rohwer et al. 2001, 2002; Koren and Rosenberg 2006; Bourne and Munn 2005). It was observed that coral-associated bacteria were species specific regardless of their geographical location (Rohwer et al. 2001; Bourne and Munn 2005). The rich species diversity of microbes on the surface of corals can be attributed to the presence of a surface mucus layer, with the composition of mucus being species specific for each coral (Meikle et al. 1988). The mucus has the ability to trap particulate organic matter from seawater and serves as an energy-rich substrate for the heterotrophic activity of bacteria (Wild et al. 2004). The mucus also aids the corals in coping up with excessive sediment stress (Hubbard and Pocock 1972) and protects them from fouling (Ducklow and Mitchell 1979).

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The nutrient-rich coral mucus supports the growth of both beneficial and pathogenic bacteria, yet little is known about the microbial population associated with corals and their pathogenicity. The microbial communities associated with corals exhibit antimicrobial properties and potentially protect corals from pathogens by preventing their colonization via competition for space on the reef and deterring their growth by releasing antimicrobial compounds (Nissimov et al. 2009; Orland and Kushmaro 2009; Rypien et al. 2010; Orland et al. 2012). Raina et al. (2009) emphasized the role of microbial communities in the biogeochemical cycling of sulfur and stated that dimethylsulfoniopropionate (DMSP) and dimethylsulfide (DMS) are potential nutrient sources for coral-associated bacteria. Shashar et al. (1994) reported the presence of nitrogen-fixing bacteria in the coral skeleton playing an important role in the nitrogen recycling in the reef ecosystem. Coral probiotic hypothesis suggested that the coral holobiont could shift as an adaptive strategy of corals to the changing environmental conditions (Reshelf et al. 2006). Recent research on coral-associated bacteria suggested two contradictory scenarios following a stress event. The first is an irreversible replacement of the surface microbial biota with potential pathogens that makes the coral deprived of antimicrobial properties associated with the original microbial community. This shuffling causes disease in corals (Jones et al. 2010). The second, proposed by Garren et al. (2009), suggests that the microbial biota replaced with pathogens due to a stress is reversible in due course of time irrespective of the subsidence of the stress and mere presence of pathogens does not cause disease in corals. This was supported by experimental evidence by Sweet et al. (2011b), in which an adjusted coral microbial community was restored back to its original composition after a time period.

Ultraviolet (UV) radiation is one of the most dominant stressors to a variety of marine organisms, affecting their health and function of the marine ecosystems (Hader et al. 2011). UV radiation acts synergistically with elevated seawater temperature to cause coral bleaching (Lesser and Farrell 2004). UV-A radiation influences the productivity of marine ecosystems by affecting interactions between organisms at different trophic levels (Ochs and Eddy 1998). Most organisms synthesize UV-absorbing compounds along with other UV-protective mechanisms like photo reactivation and other repair mechanisms (Hader et al. 2007; Rastogi and Sinha 2011; Bhatia et al. 2011). Certain reef-building corals possess UV-absorbing compounds such as mycosporine-like amino acids (MAA) that include mycosporine, palythine and palythinol which protect them from harmful UV rays (Dunlap and Chalker 1986). MAA was originally isolated from cyanobacteria and also synthesized by certain phytoplankton groups and macroalgae (Hader et al. 2011). In addition to MAA, the calcium carbonate skeleton of corals also absorbs harmful UV radiation and emits it as yellow fluorescence, significantly reducing the tissue damage (Reef et al. 2009). However, the concentration of UV-absorbing compounds decreases during a sharp rise in temperature, which in turn increases the risk of the harmful effects of UV radiation to both corals and zooxanthellae (Lesser et al. 1990). Corals get external protection from harmful UV rays by the presence of chromophoric dissolved organic matter (CDOM) in overlaying waters which absorbs harmful UV radiation; however, bleaching of the CDOM exposes corals to the harmful UV rays (Anderson et al. 2001).

In this study, UV-absorbing bacteria associated with mucus of the massive coral *Porites lutea* and the table coral *Acropora hyacinthus* were isolated, identified and characterized phylogenetically. Their in vitro UV-absorbing property under elevated temperature conditions was also investigated.

Materials and methods

Description of the study site

Mucus was collected from the corals in Palk Bay, located on the southeast coast of India (Fig. 1). Palk Bay is a shallow basin with an average depth of 9 meters, and the reef here occurs at a depth of 1–4 m. The reef itself is narrow and runs parallel to the land lying between longitudes 79°17′40″E and 79°8′E and latitude 9°17′N in an east–west direction. It is composed largely of massive boulder corals, belonging to the genera *Porites, Favia, Favites, Platygyra, Symphillia,* intermittent with sandy patches. In addition, few colonies of *Acropora sp.* can be found distributed in the reef crest.

Sample collection and isolation of bacteria

Mucus samples were collected from *Porites lutea* and *Acropora hyacinthus* during pre-summer (March) and summer seasons (May) in 2010 using sterile syringes at a depth of 3 and 4 m, respectively. The syringes were immediately transported in ice to the laboratory under aseptic condition within 2 h of collection.

The mucus samples were serially diluted (tenfold dilution) using filter sterilized and autoclaved seawater, plated on ZoBell marine agar plates and incubated at 28 °C for 48 h. After incubation, the microbial population of each plate was enumerated by counting the number of individual colonies grown using a colony counter and the number of colonies was expressed as cfu/ml of mucus. Colonies with unique morphology were further purified by quadrant

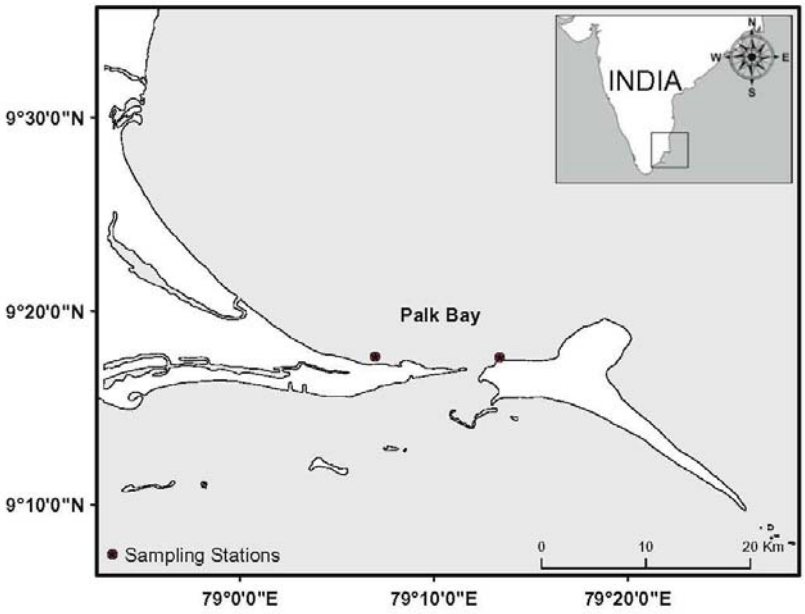

Fig. 1 Sampling sites in Palk Bay, southeast coast of India. (ArcGIS v 9)

streaking onto fresh nutrient agar plates prepared using aged seawater.

Screening of UV-absorbing bacteria

Bacterial isolates with unique morphology were inoculated individually into test tubes containing 50 ml nutrient broth prepared in aged seawater. The broth was incubated for 24 h at room temperature in an orbital shaker. After incubation, the cultures were scanned in the UV range (200–400 nm) with a double beam UV–Visible spectrophotometer (Shimadzu UV–VIS 2550, Japan) using a 5-cm path quartz cuvette against sterile nutrient broth as a reference. The isolates that showed an absorbance peak between 200 and 400 nm were selected for further studies. Each isolate was given a unique code based on the time of collection (pre-summer and summer), coral species and the number of the isolate for the ease of presenting the data, for example, PSPL1 meant pre-summer *P. lutea* isolate 1, PSAH1—pre-summer *A. hyacinthus* isolate 1, SPL1—summer *P. lutea* isolate 1 and SPH1—summer *A. hyacinthus* isolate 1.

Effect of temperature on UV absorption

The UV-absorbing bacterial isolates were inoculated in the nutrient broth prepared in the aged seawater and incubated at a gradient of temperatures (28, 30, 32 and 34° C) for a period of 24 h. After incubation, the cultures were scanned between 200 and 400 nm wavelength as described previously.

Genomic DNA extraction and 16S rRNA sequencing

The isolates that showed UV absorption were subjected to identification using 16S rRNA sequencing. Genomic DNA was extracted from the cultures grown overnight following a modified phenol–chloroform method, and the quality of the product was checked in 0.8 % agarose gel. Gene amplification was done using the forward primer 27F (5′AGAGTTTGATCCTGG CTCAG 3′; Weisberg et al. 1991) and reverse primer 1492 R (5′GGTTACCTTACGACTT 3′; Reysenbach et al. 1992). PCR was done in a thermocycler (Applied Biosystems, USA) using the following cyclic program: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were purified using polyethylene glycol precipitation as described by Liss (1980). The purified PCR products were further extended using the 27 F and 1492 R primers in a thermocycler using the following program: initial denaturation at 96 °C for 1 min followed by 30 cycles of denaturation at 96 °C for 10 s; annealing at 50 °C for 5 s, extension at 60 °C for 4 min and final extension at 60 °C for 1 min. The final extension products were cleaned using the protocol described by De and Ramaiah (2007). Sequencing was done using an ABI sequencer (ABI PRISOM model No. 3700). Forward and reverse sequences obtained were aligned using Sequencher V4.10.1. The sequences were then submitted in the GenBank. The accession numbers are GQ 281097–GQ

281107(pre-summer isolates) and KC431013–KC431018 (summer isolates).

Phylogenetic characterization

Aligned sequences were compared with the curated database of type strains of prokaryotes present in Eztaxon server (http://www.eztaxon.org; Chun et al. 2007) to determine approximate phylogenetic affiliations. 16S rRNA gene sequences were compiled and manually aligned using the ARB software package (http://www.arb-home.de) (Ludwig et al. 2004). Phylogenetic tree calculated with 16S rRNA gene sequences for all target sequences and their close relatives using the neighbor-joining (Saitou and Nei 1987) method in ARB. The neighbor-joining (NJ) tree topology was then evaluated by bootstrap analyses (Felsenstein 2004) based on 1000 re-samplings. The final phylogenetic tree was constructed using the MEGA5 (Tamura et al. 2011).

Results

Isolation and enumeration of bacteria

The culturable bacterial counts from the mucus sample of *P. lutea* and *A. hyacinthus* were 4.1×10^7 and 1.2×10^7 cfu ml^{-1}, respectively, for the pre-summer season. For the summer season, the culturable bacterial counts from the mucus sample of *P. lutea* and *A. hyacinthus* were 7×10^7 and 2.13×10^7 cfu ml^{-1}, respectively. A total of 12 isolates from *P. lutea* and 31 isolates from *A. hyacinthus* were obtained during the pre-summer season. Subsequently, a total of 13 isolates from *P. lutea* and 20 isolates from *A. hyacinthus* were obtained during the summer season.

UV-absorbing bacteria

Of the total 43 isolates obtained from *P. lutea* and *A. hyacinthus* during pre-summer season, 6 isolates from *P. lutea* and 5 from *A. hyacinthus* exhibited UV-absorbing property ($\lambda_{max} = 333$ nm; and $\lambda_{min} = 208$ nm, respectively). Only 6 isolates out of a total of 33 isolates obtained during summer season exhibited UV-absorbing property at a range of $\lambda_{max} = 282$ nm to $\lambda_{min} = 208$ nm (3 each from *P. lutea* and *A. hyacinthus*).

Phylogenetic analysis

The nearest phylogenetic neighbors of the UV-absorbing isolates were identified following BLAST analysis of 16S rRNA gene sequence. The DNA sequences exhibited <97 % identity to the nearest Eztaxon entry. 16S rRNA sequence analysis of the UV-absorbing isolates revealed the presence of two major bacterial groups. Majority of the UV-absorbing isolates were of *Firmicutes* (class *Bacilli*) and the rest were of phylum Proteobacteria (class *Gammaproteobacteria*) in both *P. lutea* and *A. hyacinthus*. Of the 11 UV-absorbing isolates obtained during the pre-summer, 8 isolates PSPL 1, PSPL 3, PSPL 4, PSPL 5, PSPL 6, PSAH 1, PSAH 2 and PSAH 3 were clustered within the *Firmicutes* group (Fig. 2) comprising the genus *Bacillus* and the rest belonged to the *Gammaproteobacteria* comprising the genus *Vibrio* (PSPL 2, PSAH 4 and PSAH 5). In contrast, all the UV-absorbing bacterial isolates obtained during summer were clustered within *Firmicutes* group comprising three different genus namely *Bacillus* (SPL 2, SPL 3, SAH 1 and SAH 2), *Staphylococcus* (SPL 1) and *Salinicoccus* (SAH 3). To compare the levels of novelty with other bacterial communities, we assembled 16S rDNA libraries from previously published data for coral-associated bacteria. The phylogenetic tree constructed to determine their affiliations (Fig. 2) formed four groups. Group A represented *Bacillus* species, the most abundant bacterial species associated with the coral mucus examined in this study. Group B and C represented *Staphylococcus* and *Salinicoccus,* respectively. The isolates related to *Bacillus kochii* (GQ281101; GQ281098; KC431016), *Bacillus gibsonii* (GQ281102) *and Bacillus oceanisediminis* (GQ281103; GQ281100) were not previously isolated from corals, and this is the first report of the isolation of these species from corals. Group D represents *Vibrio* species, the second most abundant bacterial species associated with corals in the present study. The *Vibrio sp.* (GQ281099; GQ281105; GQ281106) reported in this study was 99 % similar to *Vibrio owensii* (JX127215), which is reported to cause *Montipora* white syndrome in the Hawaiian reef coral *Montipora capitata* (Ushijima et al. 2012). Groups A, B and D were aligned with representatives of coral-associated bacteria found from previous studies, whereas group C was not, as there were no previous reports of these bacterial sp. associated with corals.

Effect of temperature on UV absorbance

For UV-absorbing isolates incubated at 28 °C, peaks of absorption wavelength were below 300 nm (ranging between 208 and 282 nm), with the exception of four isolates (PSPL 2, PSAH 4, SAH 1 and SAH 3) which showed absorption peaks above 300 nm. Incubating all the isolates at higher temperatures triggered additional absorption peaks in the UV-A range ($\lambda_{max} = 367$ nm; and $\lambda_{min} = 317$ nm). The absorption wavelengths for the UV-absorbing bacterial isolates during the pre-summer and summer seasons incubated at different temperature are presented in Figs. 3 and 4, respectively.

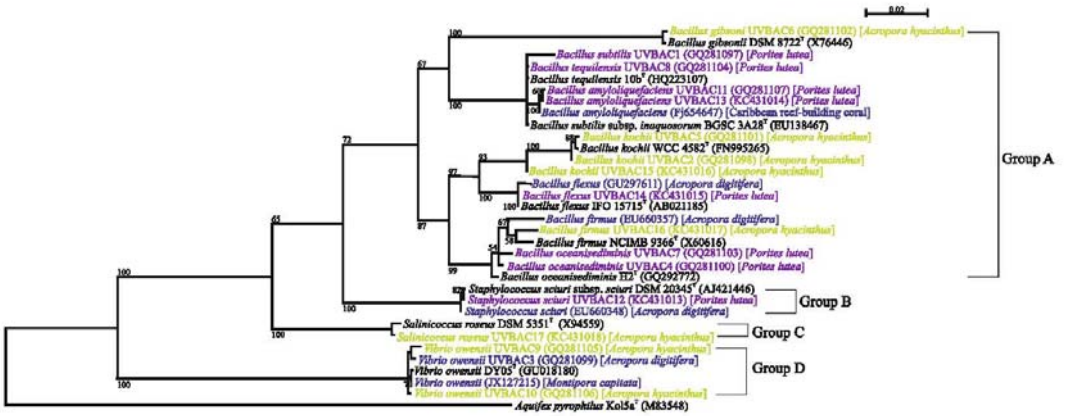

Fig. 2 Neighbor-joining (NJ) phylogenetic tree for 16S rRNA gene sequences showing the relationship between the coral-associated bacterial strains (GENBANK sequences, in *dark blue*), UV-absorbing bacterial isolates from this study [*Acropora hyacinthus* (*green*), *Porites lutea* (*purple*) and reference strains (*black*)]. The sequence of *Aquifex pyrophilus* (M83548) was used as an outgroup. Bootstrap values >50 are indicated at corresponding nodes. Sequence accession numbers are in *parentheses*

Discussion

The UV-absorbing bacterial community isolated from the mucus of *P. lutea* and *A. hyacinthus* during the pre-summer and summer seasons was dominated by *Bacillus* and *Vibrio* species. When comparing the distribution of the UV-absorbing bacterial population, *A. hyacinthus* showed a greater microbial diversity than *P. lutea* in both the pre-summer and summer seasons. However, the abundance of UV-absorbing bacteria was more or less equal between the two species.

Shallow-water corals receive higher amounts of UV radiation than those found at greater depths especially during summer seasons and they showed to possess MAAs to help protect them from UV rays. Shick et al. (1996) reported that the coral surface mucus layer offers protection against UV rays, as it consists of MAAs secreted by the algal symbionts. In addition, we reported the presence of UV-absorbing bacteria in the coral mucus, which has the potential to play an important role in protecting the corals from UV radiation.

The number of UV-absorbing bacteria isolated was significantly low relative to the other heterotrophic bacteria in coral mucus during the summer season for both *A. hyacinthus* and *P. lutea* as compared to the pre-summer season. This could potentially be attributed to the rise in seawater temperatures. When the corals were under thermal stress, the population of algal symbionts was significantly reduced which subsequently affected mucus composition and secretion (Brown and Bythell 2005). Also, Ritchie and Smith (1995) stated that the chemical nature and quantity of mucus could change when the coral was stressed. The bacterial communities outcompete one over the other during the incidence of high temperature and bleaching (Ritchie et al. 1994). In our study, the change in the composition of mucus (especially during an increase in seawater temperature), would have favored other heterotrophic bacteria to outcompete the UV-absorbing bacteria associated with the coral mucus. Currently, nothing is known about the UV-absorbing properties and numerical abundance of UV-absorbing bacteria within the un-cultured mucus-associated bacterial population, and this should be the focus of the next area of research on this topic. Rising incubation temperatures for the UV isolates from 28 to 34 °C shifted the wavelength of UV absorption to a higher range. This is of particular importance, as Gleason and Wellington (1993) state that UV-A radiation at the wavelength band of 280–400 nm is one of the major contributing factors in coral bleaching. Prevalence of these UV-absorbing bacteria during an episode of brief warming period could be an indication of the adaptive mechanism in the corals to shuffle and proliferate a particular group of bacteria that can absorb these lethal UV radiations.

It is not known whether the UV-absorbing property exhibited by the bacterial isolates from our study was due to the synthesis of UV-absorbing compounds like MAAs. MAAs (mycosporine-like amino acids) is a collective term given to a group of amino acids synthesized as secondary metabolites in the shikimic acid pathway involved in the synthesis of aromatic amino acids that protect aquatic organisms against harmful solar radiations. MAAs consists of cyclohexenone or cyclohexinimine chromophore conjugated with the nitrogen substituent of an amino acid (Sinha et al. 1998). More than 20 different MAAs have

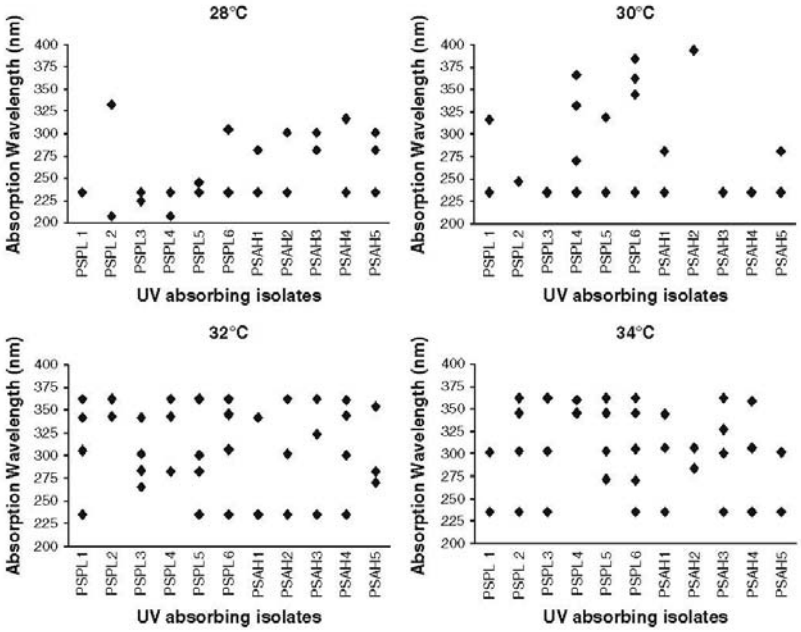

Fig. 3 Absorption wavelength of UV-absorbing bacteria isolated during pre-summer season in response to increasing temperature. With increase in temperature, additional absorption peaks were observed in the UV-A range

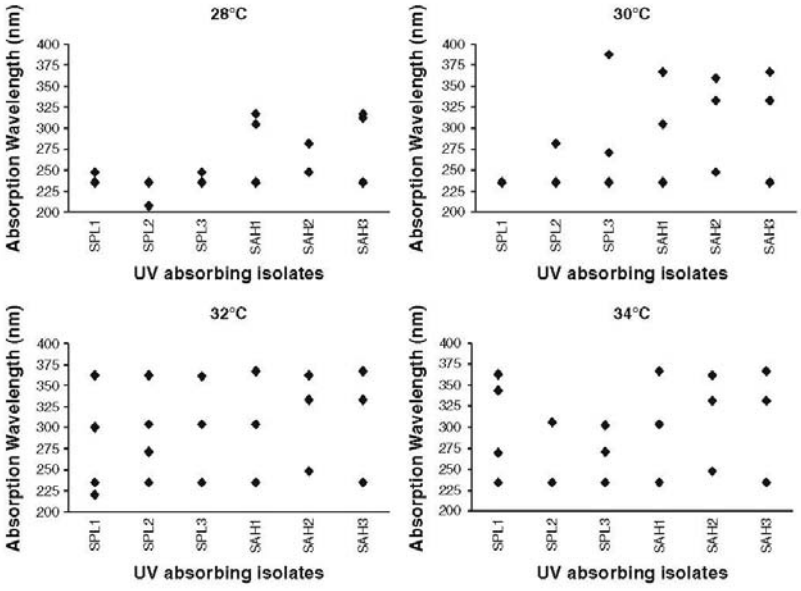

Fig. 4 Absorption wavelength of UV-absorbing bacteria isolated during summer season in response to increasing temperature

been reported in various organisms each having their own absorption maxima due to the difference in attached side groups and nitrogen substituents (Singh et al. 2008). Dunlap and Shick (1998) reviewed the presence of MAAs in reef-associated organisms that either synthesize their own MAAs or acquired them from their algal symbionts. Biosynthesis of MAAs via the shikimic acid pathway (Favre-Bonvin et al. 1987) was reported to be absent in the animal tissues (Bentley 1990), suggesting that zooxanthellae might be the source. Contrary to this, there are also

reports of zooxanthellae not being the source of MAAs (Banaszak and Trench 1995; Ishikura et al. 1997). However, in the majority of the studies, the same type of MAAs was isolated from both the zooxanthellae and host animal tissue indicating that they share this compound (Dunlap and Shick 1998).

In general, heterotrophic bacteria lack UV-protective pigments and compounds (Karentz et al. 1994), but Arai et al. (1992) isolated and characterized UV-absorbing compounds from a marine bacterium *Micrococcus*. In the study presented here, bacteria isolated from coral mucus and cultured independently under laboratory conditions do exhibit UV-absorbing properties, indicating the synthesis of UV-absorbing compounds by the bacteria itself. When these UV-absorbing bacterial isolates were incubated at temperatures higher than 28 °C, additional absorption peaks appeared in the UV-A range (315–400 nm). This may be due to the synthesis of additional UV-absorbing compounds as previously described in cyanobacteria (Sinha et al. 2001) and in the marine macroalga *Chondrus crispus* (Karsten et al. 1998). The synthesis of UV-A-absorbing compounds by these bacteria in response to elevated temperatures suggests that a rise in temperature could indirectly aid in diluting the effects of UV-A radiation on coral hosts.

In conclusion, this study suggests that the UV-absorbing bacteria associated with the coral mucus might play an important role in protecting the corals during incidences of increased UV radiation. Unraveling the complex relationship between corals and their associated bacteria will help us in understanding the adaptive strategies corals and their associates use against various environmental stresses. Moreover, in this study, only culturable bacteria were shown to absorb UV radiation. Exploring the uncultured bacteria associated with the coral mucus will show the actual magnitude of the UV protection rendered by these coral-associated bacteria.

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Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India

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2

ABSTRACT

Bacillus species constitute a diverse group of bacteria widely distributed in soil and the aquatic environment. In this study, *Bacillus* strains isolated from the coastal environment of Cochin, India were identified by detailed conventional biochemical methods, fatty acid methyl ester (FAME) analysis and partial 16S rDNA sequencing. Analysis of the data revealed that *Bacillus pumilus* was the most predominant species in the region under study followed by *B. cereus* and *B. sphaericus*. The *B. pumilus* isolates were further characterized by arbitrarily primed PCR (AP-PCR), antibiotic sensitivity profiling and PCR screening for known toxin genes associated with *Bacillus* spp. All *B. pumilus* isolates were biochemically identical, exhibited high protease and lipase activity and uniformly sensitive to antibiotics tested in this study. One strain of *B. pumilus* harboured cereulide synthetase gene *cesB* of *B. cereus* which was indistinguishable from rest of the isolates biochemically and by AP-PCR. This study reports, for the first time, the presence of the emetic toxin gene *cesB* in *B. pumilus*.

Keywords: *B. pumilus*; FAME; 16S rDNA; *cesB*; AP-PCR.

3

INTRODUCTION

The Gram-positive, aerobic, rod-shaped endospore-forming bacteria of the Genus *Bacillus* are the most widely represented organisms in the soil. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* spp. adapt easily to diverse habitats (25). The diverse physiology of *Bacillus* spp. requires elaborate biochemical tests for their identification (30). Advances in chromatographic analysis of whole cell fatty acid methyl ester (FAME) profiles have made this technique sufficiently sensitive and reliable for grouping of *Bacillus* at species level (31). Further, nucleic acid based techniques such as 16S rDNA (3,34) and gyrase B (*gyrB*) sequence analysis have proved to be of immense value for phylogenetic analysis of bacteria (35). Based on the 16S rDNA sequence analysis, 5 groups have been identified within the genus *Bacillus*, of which the group 1 (*B. subitilis* group) comprises of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* (3,12).

Several species of *Bacillus* inhabit coastal and marine environments, though it is hard to strictly classify them as indigenous to these habitats. Together with *B. cereus* and *B. subtilis*, *B. pumilus* is considered as a major component of marine bacterial communities (8,15,22,27). Recently, *B. pumilus* has also been reported to be the second most predominant *Bacillus* species in spacecrafts (17). This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H_2O_2 and chemical disinfections (19). The ecological role of *B. pumilus* is emphasized by the fact that they do produce compounds antagonist to fungal and bacterial pathogens (4,6). Thus, *B. pumilus* is of considerable research interest to understand its physiological diversity, genetic relatedness with other *Bacillus* spp. and the possible presence of toxigenic factors. In the study reported here, we describe i) isolation and

identification of *Bacillus* spp. from environmental samples by conventional methods, FAME and 16S rDNA sequencing and ii) further phenotypic and genetic characterization of *B. pumilus*, the predominant *Bacillus* group of bacteria in the coastal region under study.

MATERIALS AND METHODS

Isolation and biochemical characterization of *Bacillus* spp.

Sea water, sediment, fish and shellfish were collected off Cochin, West coast of India and processed for the isolation of *Bacillus* spp. Fish, shellfish or sediment samples were homogenized in phosphate buffered saline (PBS 0.05 M, pH 7.2), serially diluted in the same medium and spread plated on nutrient agar prepared in 50% seawater. One hundred micro liters of seawater samples were directly spread plated on the same medium and incubated at 30 ºC for 24-48 h. The colonies that came up on agar plates were purified and stored at -80ºC in nutrient broth containing 30% glycerol. For taxonomic identification, the isolates were subjected to a series of biochemical tests (11), which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, temperature and pH ranges, degradation of starch, casein, urea, tween 20, gelatin, chitin, acid production from arabinose, mannitol, xylose, glucose, lactose, citrate utilization and production of DNAse. The production of extracellular enzymes namely caseinase, chitinase, protease, alkaline phosphatase, gelatinase and lipase was studied following the protocol described by Smibert and Krieg (29)

Fatty acid methyl ester (FAME) analysis

Gas chromatographic analysis of whole cell fatty acid methyl ester (FAME) was performed for further identification and grouping of isolates. Fatty acid methyl ester extraction was performed using standard procedures (28). The fatty acid profiles generated were compared against an inbuilt Sherlock TSBA Library version 3.9 (MIDI Inc., DE, USA). A similarity index of >60% was used for clustering of isolates at species level.

Antimicrobial susceptibility assay

The inhibition of *B. pumilus* strains by various antibiotics was tested by standard disc diffusion technique (7). The cultures were grown in nutrient broth overnight and plated on Muller Hinton agar (Hi-Media, Mumbai). The following antibiotic discs with their concentrations indicated in parenthesis were used; amoxicillin (25 mcg), penicillin (10 mcg), ciprofloxacin (5 mcg), gentamycin (10 mcg), cotrimoxazole (25 mcg), chloramphenicol (30 mcg), bacitracin (8 mcg), tetracycline (30 mcg), kanamycin (30 mcg), erythromycin (15 mcg), vancomycin (30 mcg).

DNA isolation and purification

Pure genomic DNA was isolated following the method of Ausubel *et al.* (5). Briefly, the cultures were grown overnight in 3 ml nutrient broth with shaking at 30 °C. A 1.5 ml of the culture was centrifuged at 12 000 *g* for 10 min and the resultant pellet was resuspended in 567 µl 1× TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Proteinase K and SDS were added to final concentrations of 100 µg/ml and 0.5% respectively, and incubated at 37 °C for 1 h. After incubation, NaCl (5 M) and CTAB/NaCl (10% w/v cetyl trimethyl ammonium bromide in 0.7 M NaCl) were added and incubated at 65 °C for 10 min. The mixture was extracted once each with

an equal volume of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase using 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet obtained after final centrifugation was vacuum dried and dissolved in 50 µl 1× TE buffer. DNA quantification was done using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan).

16S rDNA sequencing and AP-PCR

The 16S rDNA of 4 strains (NIOB 005, NIOB 133, NIOB 485 and NIOB 525) were PCR amplified using universal primers and PCR conditions described by Iwamoto *et al.* (16) (Table 1). The resultant 454 bp products were purified using a PCR purification kit (Qiagen, Germany) and sequenced. The sequences were subjected to homology search using BLAST programme (2) of the National Center for Biotechnology Information (NCBI).

AP-PCR was performed using primer CRA22 described by Neilan (18) (Table 1). All the reactions were carried out in 30 µl volumes consisting of a 10×buffer (100 mM Tris-HCl, 500 mM KCl and 20 mM $MgCl_2$), 200 µM concentrations of each of the four dNTPs, 30 picomoles of primer, 3 U of Taq polymerase (MBI Fermentas). All PCR amplifications were carried out in an eppendorf mastercycler (Eppendorf, Germany). In all the reactions, 300 ng of the pure genomic DNA was used. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photographed. Amplification profiles obtained were analyzed and a dendrogram was generated using BioNumerics version 4.6 software (Applied Maths, Belgium)

PCR detection of cereulide synthetase genes

B. pumilus were screened for the presence of cereulide synthetase genes *cesA* and *cesB* using primers previously described (Table 1). The expected amplicons with *cesA* and *cesB* gene-specific primers were 188 bp and 635 bp respectively.

Nucleotide sequence accession numbers

The partial 16S rDNA and *cesB* sequences derived in this study have been deposited in GenBank under the accession numbers EU283326, EU283325, EU283323, EU283322, EU283320, EU283318, EU167933, EU283321, EU167932, EU167924, EU289221

RESULTS

Taxonomic identification of *Bacillus* spp. isolated from coastal environment of Cochin

Eighty-two *Bacillus* spp. were isolated and identified by biochemical tests and fatty acid methyl ester analysis (FAME). These included *B. pumilus* (16), *B. cereus* (15), *B. sphaericus* (11), *B. subtilis* (10), *B. amyloliquefaciens* (8), *B. megaterium* (6), *B. lentimorbus* (5), *B. coagulans* (4), *B. licheniformis* (4), *B. circulans* (1), *B. flexus* (1) and *Bacillus* GC group 22 (1). The *Bacillus* GC group 22 corresponds to the gas chromatographic profile of a *Bacillus* species in the Sherlock TSBA Library version 3.9 (Microbial ID, MIDI Inc.), the 16S rDNA sequence of which does not match any known species of the genus *Bacillus*. In our study, *B. pumilus* was the most predominant species followed by *B. cereus* and *B. sphaericus*. Ivanova *et al.* (15) in their study

found that *B. pumilus* and *B. subtilis* were the most abundant *Bacillus* spp. associated with marine sponges, ascidians, soft corals, and seawater.

Characterization of *B. pumilus*

Sixteen *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). Physiological tests revealed the production of detectable protease and lipase but not amylase, phosphatase, DNase, gelatinase and chitinase. The partial 16S rDNA sequences (≈500 bp) of *B. pumilus* determined in this study revealed 99-100% homology with *B. pumilus* 16S rDNA sequences in the GenBank. The antibiotic susceptibility profiles of *B. pumilus* were identical. All 16 isolates were uniformly inhibited by amoxicillin, ciprofloxacin, gentamycin, cotrimoxazole, chloramphenicol, bacitracin, tetracycline, kanamycin, erythromycin, vancomycin. All isolates exhibited resistance to penicillin.

AP-PCR typing of isolates

The random primer CRA 22, which consistently yielded 4-10 bands with *B. pumilus*, was chosen for typing of isolates by AP-PCR. The analysis of AP-PCR fingerprints revealed heterogeneity among *B. pumilus* isolates with 10 distinct patterns (Fig. 1). Despite this overall genetic diversity, near identical profiles were obtained between strains NIOB 485 and NIOB 525 (from crab), strains NIOB111 (fish) and NIOB 426 (from sediment), strains NIOB096 (from crab) and NIOB169 (from fish), strains NIOB163 (from fish) and NIOB431 (from starfish).

Detection of cereulide synthetase gene in *B. pumilus* by PCR

One isolate *B. pumilus* NIOB 133 isolated from an estuarine fish yielded 635 bp amplicon with *cesB*-targeted primers EM1-f and EM1-r (Fig. 2). The product of *cesB* PCR was sequenced and nucleotide sequence analysis of the PCR product revealed 96% similarity with corresponding sequence of *cesB* of *B. cereus* (GenBank accession no. DQ889676) (26), while the deduced amino acid sequence showed 92% homology with a few amino acid mismatches (Fig. 3). This strain was negative by *cesA* PCR using primers CER1 and EMT1.

DISCUSSION

This study on the diversity of *Bacillus* spp. isolated from a coastal environment by biochemical assays, FAME analysis and 16S rDNA sequencing revealed that *B. pumilus* was the predominant species followed by *B. cereus*. *B. pumilus* belongs to *B. subtilis* group of aerobic spore-forming organisms, which has lately evoked considerable research interest due its involvement in cases of food-poisoning. Recently, a pumilacidin-producing *B. pumilus* has been implicated in a case of food poisoning (13). A study by Brophy and Knoop (9) reported experimental induction of enterocolitis in guinea pigs, while some compounds produced by *B. pumilus* were reportedly toxic to mice, eukaryotic cells and humans (20,21,24,32).

Sixteen environmental *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). The identity of these isolates was further confirmed by fatty acid methyl ester analysis with a similarity index of >60% (data not shown). The partial 16S rDNA sequences (≈500 bp) of these strains revealed 99-100% similarity with *B. pumilus*

16S rDNA sequences in the GenBank. Thus, a combination of conventional physiological tests and genetic analysis enabled unambiguous identification of *B. pumilus* from coastal environments of Cochin. None of the strains exhibited resistance to the antibiotics tested in this study except to penicillin. This observation is interesting, since penicillin resistance has not been reported in *B. pumilus*. Studies on antibiotic resistance of *B. pumilus* are limited, since the organism is not considered infectious to humans and animals. However, some recent studies have revealed that several *Bacillus* species including *B. pumilus* can cause infections, ranging from skin infection to life threatening bacteremia in immunocompromised individuals (23,33). Thus, more studies need to be performed to understand the human health significance of *B. pumilus*, genetic basis of infections and resistance to antimicrobials.

The whole genome comparison of *B. pumilus* strains by AP-PCR demonstrated that *B. pumilus* were genetically diverse. Though the isolates shared several common amplification bands, overall heterogeneity among *B. pumilus* studied was apparent (Fig. 1). Despite this, some strains isolated from similar sources during different points time exhibited identical or near identical profiles. However, it was not possible to attribute isolates to a particular source solely based on the AP-PCR profiles, since some strains isolated from different sample types such as fish and sediment also exhibited identical AP-PCR profiles. The random primer CRA 22 used in this study has sufficient discriminating power and will be useful for studying genetic diversity among the *Bacillus* group of bacteria.

In order to understand the toxigenic potential of environmental strains of *B. pumilus*, the isolates obtained in this study were screened for the presence of cereulide synthetase genes *cesA* and

cesB by PCR. Surprisingly, one strain (NIOB 133) was PCR positive for *cesB*-targeted gene (Fig. 2). This strain however, did not harbour *cesA* gene as revealed by *cesA*-specific PCR using primers CER1 and EMT1. Cereulide is a small heat stable cyclic dodecadepsipeptide produced by some strains of *B. cereus* which has high toxicity to humans (1,36). Cereulide is synthesized by a non-ribosomal peptide synthetase, encoded by the *ces* genes located on a 270-kb pXOI-like virulence plasmid named pCER270 (10,26), more often found associated with clinical isolates of *B. cereus*. A recent study has reported the presence of *cesA*, but not *cesB*, in *B. pumilus* and *B. licheniformis* isolated from bovine mastitis (20). However, the *cesB*-positive strain in our study lacked *cesA*. Therefore, it appears that cereulide-encoding genes may be distributed over a wide species range within the *Bacillus* group of bacteria in the environment. The *cesB*-positive *B. pumilus* in the present study was indistinguishable from the rest of the strains by biochemical assays or by its fatty acid profile. Further, the AP-PCR profile of this strain was similar to other non-toxigenic strains (Fig. 1). The production of toxin and the consequent ability of this strain to initiate emetic symptoms need to be established by suitable animal feeding experiments. Our study constitutes the first report on the presence of *cesB* in *B. pumilus*.

In conclusion, *Bacillus* spp. constitute key components of coastal-marine heterotrophic bacterial communities owing to their diverse and flexible physiological properties. Isolation and characterization of *Bacillus* spp. from these environments will help in identifying novel mechanisms of environmental survival, diverse metabolic activities, production of biotechnologically valuable compounds such as enzymes and antimicrobial substances and the presence of putative toxigenic factors. In our study, a combination of methods involving FAME, 16S rDNA sequencing and biochemical assays enabled complete identification of *B. pumilus*.

Though, the biochemical and enzymatic properties of *B. pumilus* isolated in this study were uniform, intraspecific genetic diversity was evident from AP-PCR analysis. The detection of cereulide synthetase gene *cesB* in *B. pumilus* is significant, since *ces* genes were previously thought to be restricted to emetic strains of *B. cereus*. The organization of *ces* operon in *B. pumilus* is a subject for further study.

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13

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17

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18

Table 1. PCR primers used in this study

Primer	Sequence (5'-3')	Target gene	Reference
EUB-F	gcacaagcggtggagcatgtgg	16S rDNA	16
EUB-R	gcccgggaacgtattcaccg		
CER1	atcataaaggtgcgaacaaga	Cereulide synthetase (*cesA*)	14
EMT1	aagatcaaccgaatgcaactg		
EM1-F	gacaagagaaatttctacgagcaagtacaat	Cereulide synthetase (*cesB*)	10
EM1-r	gcagccttccaattactccttctgccacagt		
CRA22	ccgcagccaa	Random primer	18

19

Table 2. Sources of *B. pumilus* strains isolated in this study

Strain	Source
NIOB005	Sediment
NIOB014	Sediment
NIOB018	Sediment
NIOB026	Oyster
NIOB096	Crab
NIOB111	Fish
NIOB113	Fish
NIOB133	Fish
NIOB137	Fish
NIOB163	Fish
NIOB169	Fish
NIOB190	Fish
NIOB426	Sediment
NIOB431	Starfish
NIOB485	Crab
NIOB525	Crab

20

Table 3. Growth and substrate utilization characteristics of *B. pumilus* observed in this study.

Characteristic	*B. pumilus* phenotype	Characteristic	*B. pumilus* phenotype
Amylase	+	Substrate utilization	
Protease	+	D-Glucose	+
Lipase	+	L-Arabinose	+
Phosphatase	-	D-Xylose	+
DNase	-	D-Mannitol	+
Gelatinase	-	Galactose	+
Chitinase	-	Fructose	+
Growth temperature	5-50 °C	Mannose	+
Growth ph	5-11	Nitrate	-
Nacl tolerance	10%	Adonitol	-
Oxidase	+	Dulcitol	-
Catalase	+	Sorbitol	-
Indole production	-	Inositol	-
Voges-Proskauer	+	Urea	-
Citrate utilization	-		

21

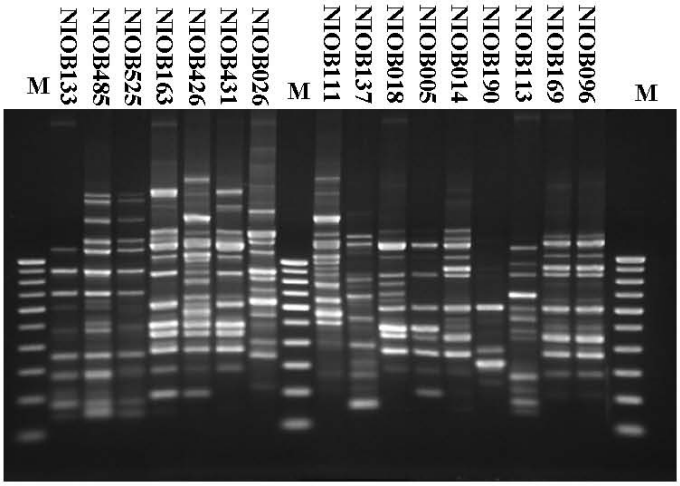

Figure 1. AP-PCR patterns of *B. pumilus* obtained with primer CRA22. M= 100 bp DNA ladder (GeneRuler, Fermentas).

22

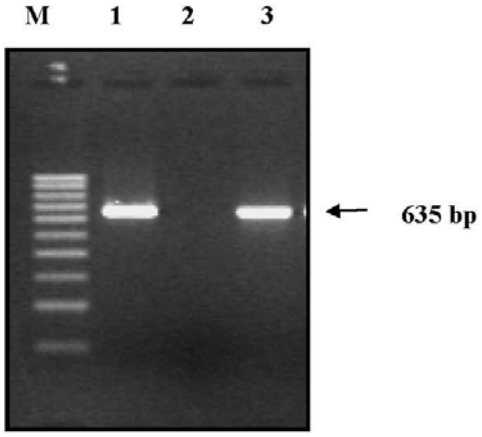

Figure 2. Detection of cereulide synthetase gene *cesB* in *B. pumilus* by PCR using primers EM1-F and EM1-r.

M= 100 bp DNA ladder (GeneRuler, Fermentas). 1: *B. pumilus* NIOB133 *cesB*$^{+}$; 2: *B. pumilus* NIOB 137 *cesB*$^{-}$; 3: *B. cereus* NIOB 020 (*cesB*$^{+}$ reference strain, environmental isolate).

23

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DQ889676 AHDIALNWMPLEHVGRIVMFHIKDTYLGRNQVQVRTQYVLSEPTRWLDLITTYKTTITWA
EU289221 ..............G.....................................A.....

         PHFAVALINKEIENGVKGSLDLLSIEFIAIAGEAINGYTAKKFLQVLSPYGLPEDAMIPV
         .N..F.............NW..S.M...VN.............................

         WGMS
         ....
```

Figure 3. Alignment of deduced partial amino acid sequence of *cesB* derived in this study from *B. pumilus* strain NIOB 133 (EU289221) with the corresponding GenBank sequence of plasmid pCER270 (DQ889676). Dots indicate identical amino acids.