

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

Jiya Jose,² Rajesh Giridhar,^{2,3} Abdulaziz Anas,² Loka Bharathi, P.A.,¹ Shanta Nair^{1*}

1. National Institute of Oceanography (CSIR), Dona Paula, Goa, India 403004
2. National Institute of Oceanography (CSIR), Regional Centre, PB 1913, Cochin, Kerala, India 682018
3. Present address: University of Texas, Department of Biology, San Antonio-Texas 78249

* Corresponding author: email. shanta@nio.org Phone:+91 832 2450239

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 metal resistance against 5mM 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 and frequency of expression for amylase, protease, gelatinase and lipase. The difference in enzyme expression profile was more prominent among the bacteria isolated from sediment. 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.

Key words: Heavy metal pollution, Bacterial diversity, Enzyme profile, adaptation

Capsule: Heavy metal pollution exerts pressure on the diversity and enzyme expression profile of estuarine bacteria

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; Bonget 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; Hoostalet 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, 1984 ; McGrath et al., 2001; Lasat, 2002; Liet al., 2006; Souza et al., 2006; Wanget 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 N₂O 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 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 extra-cellular 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 ($9^{\circ} 30'$ – $10^{\circ} 12'N$ and $76^{\circ} 10'$ - $76^{\circ} 29'E$). 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 ($9^{\circ} 58'N$) and Azhikode ($10^{\circ} 10'N$) (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 bar mouth of Cochin estuary were selected. The positions of the sampling stations, Eloor, Vypin and Munambam are shown in Figure 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 bar mouth 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 $4^{\circ} C$ 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-HClO₄-HNO₃, 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 Trypton 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; Tanget et al., 1998).

2.3. 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 HNO₃ 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 (ZnSO₄.7H₂O, HgCl₂, CoCl₂.6H₂O, CdCl₂, CuSO₄.5H₂O, NiCl₂) was poured in to the central well of PYT80 plates and was incubated at 28±2°C for 24 hours 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.

2.4. 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.

2.5. 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 (Venugopalet 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 *P. aeruginosa* without any previous exposure to heavy metals was subjected to heavy metal shock (Teitzelet al., 2006; Martinez, et al., 2009). Inova 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'Annoet al., 2003). Figure 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 enzyme expression of certain enzyme of same bacteria in different stations or the heavy metal actually selected different bacteria with different enzymatic degradation capability. 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 (Weisset al., 1991; Hoppeet 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

Figure 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 5mM 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; Deet 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; Ramoset al., 2002; Pumbweet et al., 2007; Martinez, et al., 2009).

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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6. References

- Bailey, N., 1995. Statistical methods in biology. Cambridge University Press, Cambridge, UK.
- Balachandran, K.K., Laluraj, C.M., Martin, G.D., Srinivas, K., Venugopal, P., 2006. Environmental analysis of heavy metal deposition in a flow-restricted tropical estuary and its adjacent shelf. *Environmental Forensics* 7, 345 - 351.
- Balachandran, K.K., Laluraj, C.M., Nair, M., Joseph, T., Sheeba, T., Venugopal, P., 2005. Heavy metal accumulation in a flow restricted, tropic estuary. *Estuarine Coastal and Shelf Science* 65, 361 - 370.
- Balachandran, K.K., Reddy, G.S., Revichandran, C., Srinivas, K., Vijayan, P.R., Thottam, T.J., 2008. Modelling of tidal hydrodynamics for a tropical ecosystem with implications for pollutant dispersion (Cochin estuary, Southwest India). *Ocean Dynamics* 58, 259 - 273.
- Barkay, T., Miller, S.M., Summers, A.O., 2003. Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews* 27, 355 - 384.
- Bong, C.W., Malfatti, F., Azam, F., Obayashi, Y., Suzuki, S., 2010. The effect of Zinc exposure on the bacteria abundance and proteolytic activity in seawater, in: Hamamura, N., Suzuki, S., Mendo, S., Barroso, C.M., Iwata, H., Tanabe, S. (Eds.), *Interdisciplinary studies on Environmental Chemistry - Biological responses to contaminants*. Terrapub, pp. 57 - 63.

- Chakravarty, R., Banerjee, P.C., 2008. Morphological changes in an acidophilic bacterium induced by heavy metals. *Extremophiles* 12, 279 - 284.
- Chrost, R.J., Rai, H., 1994. Bacterial secondary production in: Overbeck, J., Chrost, R. (Eds.), *Microbial ecology of lake Plusssee (Ecological studies)*. Springer - Verlag, Newyork, pp. 92 - 117.
- De, J., Ramaiah, N., Mesquita, A., Verlekar, X.N., 2003. Tolerance to varioius toxicants by marine bacteria highly resistant to mercury. *Marine Biotechnology* 5, 185 - 193.
- Dell' Anno, D., Mei, M.L., Ianni, C., Danovaro, R., 2003. Impact of bioavailable heavy metals on bacterial activities in coastal marine sediments. *World Journal of Microbiology & Biotechnology* 19, 93 - 100.
- Diaz-Ravina, M., Baath, E., Frostegard, A., 1994. Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. *Applied and Environmental Microbiology* 11.
- Dickinson, R.E., Cecerone, R.J., 1986. Future global warming from atmospheric trace gases. *Nature* 319, 109 - 115.
- Förstner, U., Wittmann, G.T.W. (Eds.), 1979. *Metal Pollution in the Aquatic Environment*. Springer Verlag Berlin Heidelberg, New York.
- Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Kreig, N.R., Philips, G.B., 1981. *Manual methods for general bacteriology* American Society for Microbiology, Washington DC.
- Haferburg, G., Kothe, E., 2007. Microbes and metals: interactions in the environment. *Journal of Basic Microbiology* 47, 453 - 467.
- Haferburg, G., Kothe, E., 2010. Metallomics: lessons for metalliferous soil remediation. *Applied Microbiology and Biotechnology* 87, 1271 - 1280.
- Hantke, K., 2001. Bacterial zinc transporters asnd regulators. *BioMetals* 14, 239 - 249.
- Hassen, A., Saidi, N., Cherif, M., Boudabous, A., 1998. Resistance of environmental bacteria to heavy metals. *Bioresource Technology* 64, 7 - 15.
- Hoostal, M.J., Gabriela Bidart-Bouzat, M., Bouzat, J.L., 2008. Local adaptation of microbial communities to heavy metal stress in polluted sediments of lake Erie. *FEMS Microbiology and Ecology* 65, 156 - 168.
- Hoppe, H.G., Arnosti, C., Herndl, G.F. (Eds.), 2002. *Ecological significance of bacterial enzyme in the marine environment*, New York.
- Ivanova, E.P., Gorshkova, N.M., Kurilenko, V.V., 2001. Tolerance of marine proteobacteria of the genera *Pseudoalteromonas* and *Alteromonas* to heavy metals. *Microbiology* 70, 239 - 241.

- Konopka, A., Zakharova, T., 1999. Quantification of bacterial lead resistance via activity assays. *Journal of Microbiological Methods* 37, 17 - 22.
- Kosolapov, D.B., Kuschik, P., Vainshtein, M.B., Vatsourina, A.V., Wiebner, A., Kastner, M., Muller, R.A., 2004. Microbial processes of heavy metal removal from carbon-deficient effluents in constructed wetlands. *Engineering in Life Sciences* 4, 403 - 411.
- Lasat, M.M., 2002. Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality* 31, 109 - 120.
- Li, Z., Xu, J., Tang, C., Wu, J., Muhammad, A., Wang, H., 2006. Application of 16S rDNA-PCR amplification and DGGE fingerprinting for detection of shift in microbial community diversity in Cu-, Zn-, and Cd-contaminated paddy soils. *Chemosphere* 62, 1374-1380.
- Lock, K., Janssen, C.R., 2005. Influence of soil zinc concentrations on zinc sensitivity and functional diversity of microbial communities. *Environmental Pollution* 136, 275 - 281.
- Loring, D.H., Rantala, R.T.T., 1977. Geochemical analysis of marine sediments and suspended particulate matter, Fisheries and marine service technical report. Environment, Canada.
- Martinez, J.L., Sanchez, M.B., Martinez-Solano, L., Hernandez, A., Germendia, L., Fajardo, A., Alvarez-Ortega, C., 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiology Reviews* 33, 430 - 449.
- McGrath, S.P., Zhao, F.J., Lombi, E., 2001. Plant and rhizosphere processes involved in phytoremediation of metal contaminated soils. *Plant and Soil* 232, 207 - 214.
- Minagawa, N., Zumft, W.G., 1988. Cadmium-copper antagonism in the activation of periplasmic nitrous oxide reductase of copper-deficient cells from *Pseudomonas stutzeri*. *BioMetals* 1, 117-122.
- Nair, M., Jayalakshmi, K.V., Balachandran, K.K., Joseph, T., 2006. Bioaccumulation of toxic metals by fish in a semi-enclosed tropical ecosystem. *Environmental Forensics* 7, 197 - 206.
- Nakagawa, S., Takaki, Y., Shimamura, S., Reysenbach, A.-L., Takai, K., Horikoshi, K., 2007. Deep-sea vent e-proteobacterial genomes provides insights into emergence of pathogens. *Proceedings of the National Academy of Sciences of the USA* 104, 12146 - 12150.
- Nies, D.H., 1999. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* 51, 730 - 750.
- Noah, N.M., Oomori, T., 2006. Evaluation of heavy metal pollution on the coastal marine environments of Okinawa island, Japan. *Bulletin of Faculty of Science University of Ryukyus* 81, 93 - 104.
- Oliver, J., Smith, R.G., 1982. Intestinal microflora of deep-sea animals: A taxonomic study. *Deep-Sea Research* 29, 785 - 794.

- Osterhout, G.J., Shull, V.H., Dick, J.D., 1991. Identification of clinical isolates of Gram-negative nonfermentative bacteria by an automated cellular fatty acid identification system. *Journal of Clinical Microbiology* 29, 1822 - 1830.
- Pennanen, T., Frostegard, A., Fritze, H., Baath, E., 1996. Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. *Applied and Environmental Microbiology* 62, 420 - 428.
- Pumbwe, L., Skilbeck, C.A., Wexler, H.M., 2007. Induction of multiple antibiotic resistance in *Bacteriodes fragilis* by benzene and benzene-derived active compounds of commonly used analgesics, antiseptics and cleaning agents. *Journal of Antimicrobial Chemotherapy* 60, 1288 - 1297.
- Ramaiah, N., De, J., 2003. Unusual rise in mercury-resistant bacteria in coastal environs. *Microbial Ecology* 45, 444 - 454.
- Ramos, J.L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-González, M.I., Rojas, A., Terán, W., Segura, A., 2002. Mechanisms of solvent tolerance in Gram-negative bacteria, pp. 743-768.
- Shannon, C.E., Weaver, W. (Eds.), 1949. *The mathematical theory of communication*. University of Illinois press Urbana, Illinois.
- Silver, S., Phung, L.T., 1996. Bacterial heavy metal resistance: new surprises. *Annual Reviews in Microbiology* 50, 753 - 789.
- Silver, S., Phung, L.T., 2005. A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *Journal of Industrial Microbiology and Biotechnology* 32, 587 - 605.
- Silver, S.M., T K, 1984 Bacterial transformations of and resistances to heavy metals. *Basic Life Science* 28, 23-46.
- Simbert, R.M., Krieg, N.R., 1981. *General characterization*. American Society for Microbiology, Washington DC.
- Smith, R.G., Windom, H.L., 1972. Analytical handbook for the determination of As, Cd, Co, Cu, Fe, Mn, Ni, Hg and Zn in the marine environment, Technical Report 72 - 76. Georgia Marine Science Center.
- Sobolev, D., Begonia, M.F., 2008. Effects of heavy metal contamination upon soil microbes: lead induced changes in general and denitrifying microbial communities as evidenced by molecular markers. *International Journal of Environmental Research and Public Health* 5, 450 - 456.
- Souza, M.-J.D., Nair, S., Lokabharathi, P.A., Chandramohan, D., 2006. Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters. *Ecotoxicology* 15, 379 - 384.

- Tang, Y.-W., Ellis, N.M., Hopkins, M.K., Smith, D.H., Dodge, D.E., Persing, D.H., 1998. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic Gram-negative Bacilli. *Journal of Clinical Microbiology* 36, 3674 - 3679.
- Teitzel, G.M., Geddie, A., De Long, S.K., Krisits, M.J., Whiteley, M., Parsek, M.R., 2006. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *Journal of Bacteriology* 188, 7242 - 7256.
- Thyagarajan, G., 2004. pollution threat to the island of Eloor: Public interest petition filed by Kerala state unit of PUCL before the monitoring committee on hazardous waste and hazardous chemicals set up by the supreme court of India. Monitoring Committee on hazardous wastes and hazardous chemicals set up by the Supreme Court of India.
- Valsecchi, G., Gigliotti, C., Farini, A., 1995. Microbial biomass, activity, and organic matter accumulation in soils contaminated with heavy metals. *Biology and Fertility Soil* 20, 253 - 259.
- Venugopal, P., Saraladevi, K., Remani, K.V., Unnithan, R.V., 1982. Trace metal levels in the sediments of the Cochin backwaters. *Mahasagar e Bulletin of National Institute of Oceanography* 15, 205 - 214.
- Wang, Y., Shi, J., Wang, H., Lin, Q., Chen, X., Chen, Y., 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. *Ecotoxicology and Environmental Safety* 67, 75-81.
- Weiss, M.S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., Schulz, G.E., 1991. Molecular architecture and electrostatic properties of a bacterial porin. *Science* 254, 1627 - 1630.
- Wilson, D.O., Reisenauer, H.M., 1970. Effect of manganese and zinc ions on the growth of *Rhizobium*. *Journal of Bacteriology* 103, 729 - 732.
- Yamada, N., Suzumura, M., 2010. Effects of seawater acidification on hydrolytic enzymes. *Journal of Oceanography* 66, 233 - 241.

7. Tables

Table 1. Concentration of heavy metal pollutants in the water and sediment samples collected from different sampling locations of Cochin Estuary (BDL – Below detectable limit)

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

Table 2. Percentage variation in the distribution of culturable bacteria isolated from water and sediment samples of Vypin, Munambam and Eloor (ND-Not detected)

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
Bacteroidetes	ND	7.7	00	7.7	4.8	ND

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						
<i>Acinetobacter baumannii</i>	-	+	-	+	+	-
<i>Chromobacterium violaccum</i>	+	-	+	-	-	-
<i>Citrobacter koseri</i>	-	-	-	+	-	-
<i>Comamonas acidovorans</i>	-	-	+	+	-	+
<i>Comamonas testosterone</i>	-	+	+	+	-	+
<i>Kluyvera ascorbata</i>	+	-	-	+	+	-
<i>Listonella anguillarum</i>	+	-	+	+	+	+
<i>Proteus mirabilis</i>	-	-	-	-	+	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+	+	+
<i>Pseudomonas huttiensis</i>	-	+	+	+	+	+
<i>Serratia marcescens</i>	-	-	+	+	-	+
<i>Shewanella pute faciens</i>	-	-	+	+	-	+
<i>Vibrio cholera</i>	-	-	+	-	+	+
<i>Vibrio furnissi</i>	+	-	+	-	+	+
<i>Xanthomonas arboricola-pruni</i>	-	-	+	+	-	+
<i>Yersinia pseudo tuberculosis</i>	-	-	+	+	-	+
Firmicutes						
<i>Bacillus cereus</i>	+	-	-	+	-	-
<i>Bacillus filicolonicus</i>	+	+	+	+	-	-
<i>Bacillus marinus</i>	+	+	-	-	+	-
<i>Bacillus mycoides</i>	+	+	-	+	+	-
<i>Bacillus oleronius</i>	+	+	-	-	+	-
<i>Bacillus pumilus</i>	+	+	+	+	+	+
<i>Bacillus sphaericus</i>	+	+	-	+	-	-
<i>Bacillus sp</i>	+	-	-	-	+	-
<i>Exigno bacterium acetylicum</i>	-	-	-	+	-	-
<i>Paenibacillus larvae pulvifaciens</i>	+	-	-	+	-	-
<i>Staphylococcus gallinarum</i>	+	-	+	-	-	+
Actinobacteria						
<i>Cellulomonas turbata</i>	+	-	+	+	+	-
Bacteroidetes						
<i>Myroides odoratus</i>	-	+	-	+	+	-
Shannon H'	2.788	2.458	2.774	3.087	2.692	2.66

Supplementary data

Table S1. Comparison of the enzyme profile of metal resistant *Pseudomonas aeruginosa* and *Bacillus pumilus* isolated from different sampling sites

Bacterial sp.	Enzymes	Enzyme activity measured as diameter of clearing zone (mm)					
		Vypin		Munambam		Eloor	
		Water	Sediment	Water	Sediment	Water	Sediment
<i>Pseudomonas aeruginosa</i>	Amylase	10	13	15	17	7	06
	Protease	14	22	12	20	10	08
	Lipase	09	11	08	09	00	00
	Gelatinase	13	18	12	18	00	00
<i>Bacillus pumilus</i>	Amylase	16	23	11	20	08	07
	Protease	18	22	19	25	10	06
	Lipase	09	12	07	12	00	00
	Gelatinase	00	00	00	00	09	07

8. Figure captions

Figure 1. Map showing study area and station locations

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

Figure 3. Percentage of metal resistant bacteria isolated from water (A) and sediment (B) samples of Vypin (blue), Munambam (red) and Eloor (Green)

Figure 1. Jiya et al

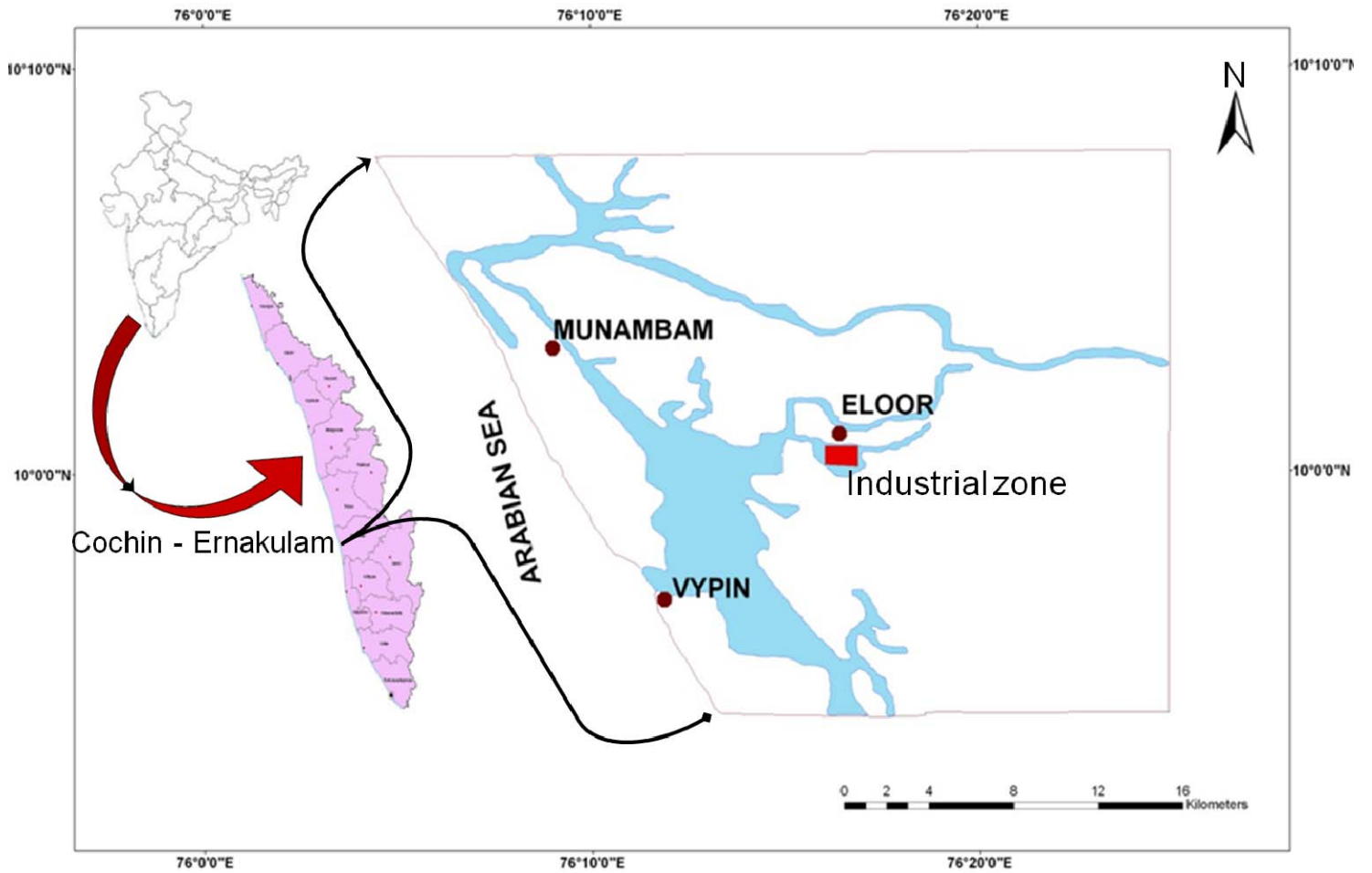


Figure 2. Jiya et al

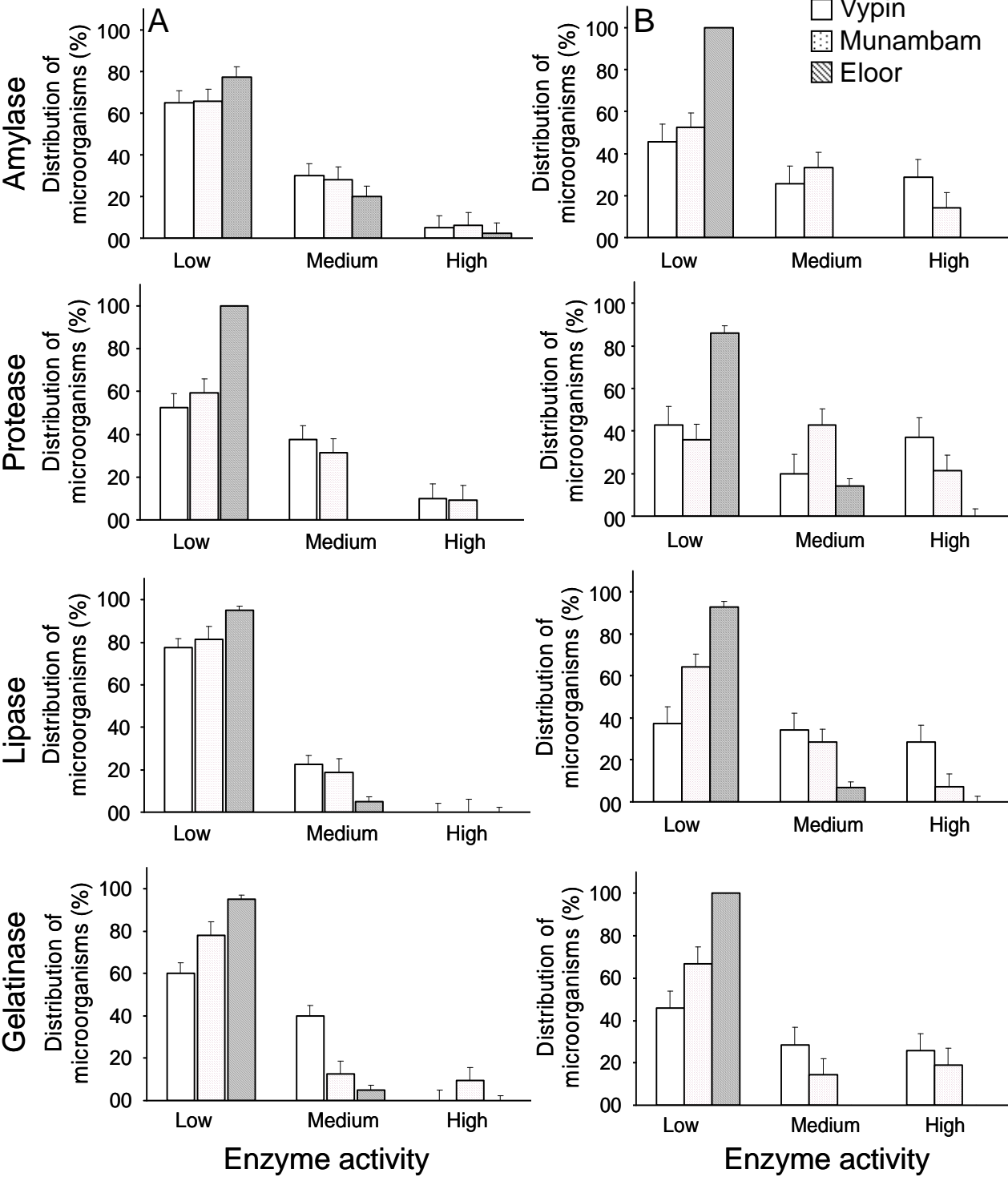


Figure 3. Jiya et al

