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Research Article

Screening for Total and Copper Resistant Bacteria Populations in the Surface Sediments in the Inner Shelf of Bay of Bengal off Chennai

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Abstract

Environmental pollution with heavy metals has accelerated dramatically since the beginning of the industrial revolution. People thought that the sea is vast and it can assimilate the wastes by dilution and can sustain the marine environment. However, continued input of the wastes has increased the concentration of deleterious components in the wastes and the marine ecosystem is under threat by anthropogenic activities. Hence an attempt has been made in the present study is to understand the impact of seasonal variation of copper resistant bacteria diversity in the coastal zone of Chennai, India. High number of metal resistance bacterial isolates were scored in ART and was followed by ET > FHT > CRT > CHT. The river mouth traverses ART and CRT scored lesser number of populations than harbor regions. The results of the present study clearly showed the increase in the development of metal resistance among the bacterial population during the study period.

Keywords: Environmental pollution, Bacterial copper resistant, Bay of Bengal, Microbial diversity, anthropogenic impact.

INTRODUCTION

Microorganisms like bacteria, fungi, algae and yeast are known to tolerate and accumulate heavy metals. Heavy metals may exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions, or modifying the active conformations of biological molecules (Moffett et al. 2002). A thorough knowledge on the microflora in the light of different metallic ion concentrations at different geophysical conditions will help to tackle not only in the conservation of ecosystem but also will enhance the utility of these microbes in handling some environmental problems. The anthropogenic inputs hence, impart severe stress on the marine ecosystem which nurtures a multitude of marine flora, which acts a natural recycling agent. The decomposers especially the heterotrophic bacteria and fungi play a major role in mineralizing and cycling of nutrients and energy from the organic wastes dumped into the oceans through domestic sewage and industrial effluents. Numerous studies have examined the heavy metal sensitivity or resistance of bacteria isolated from different habitats and many microorganisms showed adaptations to the toxic metals to which they are exposed (Müller *et al.*, 2001a). Further, the frequency of resistant bacteria to specific heavy metals may be correlated with increasing loads of metals in the environment. As a result, heavy metal resistant bacteria may be used as biological monitors or bio-indicators of environmental contamination (Baath *et al*., 1998; Richards *et al*., 2001).

Bio-indicators have been shown to be a sensitive and reliable tool in detecting the sub-lethal toxicity of these polluting compounds (Trevors *et al.*, 1985).

Microorganisms that showed adaptations to the toxic materials are reported to develop strategies to resist, tolerate, metabolize, and to detoxify these toxic substances. Due to their ability to produce bioactive compounds and their role on bioremediation of heavy metals, marine microbial group is the subject of interest for scientists. The present study aimed to isolate, identify and screening potent heavy metal resistant marine microbes from the surface sediments in the inner shelf of Bay of Bengal, off Chennai, India that could be exploited for solving environmental problems. The contaminating effect of heavy metals in the marine environment increased considerably leading to the development of high metal resistance among the marine microbes and also extending the level of metal tolerance organisms in the marine ecosystems.

MATERIALS AND METHODS

Collection of samples

Surface sediment samples were collected from 26 stations along 5 traverses, viz., Ennore Traverse (Korttalaiyar River) (ET), Fishing Harbor Traverse (FHT), Chennai Harbor Traverse (CHT), Cooum River Traverse (CRT) and Adyar River Traverse (ART) in Bay of Bengal off Chennai, India during cruise programme by CRV (Coastal Research Vessels) Sagar Purvi and Sagar Paschimi, NIOT (National Institute of Ocean Technology), MOES (Ministry of Earth Sciences), India at >10 m water depth and for shallow waters by engaging fiber glass boat during Pre-monsoon (PRM) (April - June), Monsoon (MON) (July to September) and Post monsoon (POM) (October - December) seasons of three consecutive years of 2006-07, 2007-08 and 2008-09 using Van Veen Grab sampler. Position fixing was done using onboard GPS (Global Positioning System). The samples were kept cool in an icebox during transportation to the laboratory.

Isolation and screening of microorganisms

Sediment samples (1g) were serially diluted and 10^{-4}, 10^{-5} and 10^{-6} dilutions were used for screening total and copper resistant bacteria (CuRB) populations. The dilutions were plated on copper amended sea water nutrient agar (SWNA) medium. Based on the previous studies from Pulicat Lake sediments, near Chennai coast (Kamalakannan *et al.*, 2006), basic concentration of 0.3 mM of $CuSO_4$ (Copper sulphate) was selected for the isolation of metal resistant bacteria from sediments samples. Nutrient media prepared using aged seawater was used for the isolation of bacteria. Inoculated petri plates were incubated at 35°C for 48 h and the colonies developed were counted. Bacterial colonies developed on the plates after 2 days of incubation. Morphologically distinguished colonies were purified by repeated quadrant streaking on solid SWNA plates. The purified colonies were stored in slants as well as in glycerol stocks (25% v/v) at -40ºC for further studies. The cultures were maintained by sub-culturing at regular interval of one month. The selected bacterial strains was characterized by subjecting to routine microbiological and biochemical tests (Cappuccino and Sherman, 1999) and identified using the key provided by Bergey’s Manual of Determinative Bacteriology (1994).

RESULTS AND DISCUSSIONS

Total bacterial population in surface sediments

Bacteria isolated from natural habitats show metal tolerance and multi metal resistance, which provide them selective advantages. Bacterial populations in sea sediments off Chennai differed greatly with the sampling stations. Total bacterial population during three seasons of three consecutive years, revealed no significant difference among the seasons, in some cases, the POM samples exhibited slightly higher population than the rest of the seasons and the trend observed among the traverses studied was FHT > CHT > ET > ART > CRT. The near sea shore sediment samples exhibited higher bacterial population and it was decreased with increasing the depth area and it was fallowed all the traverses of the study (Table 1).

In the present study, bacterial population recorded from the surface sediment samples during all seasons and traverses was in the range of 4.5 to 6.2 log10 (0.3 to 15.6 X 10^5) CFU/g sediment with an average of 5.4 log10 (4.2 X 10^5) CFU/g of sediment. Das *et al.*, (2007) who have collected sediment samples from the continental slope of Bay of Bengal, India recorded the total heterotrophic bacterial population counts that ranged from 0.42 to 3.74 X 10^5 cfu/g sediment. The bacterial population recorded in different coastal area of Bay of Bengal, viz., Karaikkal (4.4 X 10^4 cfu/g), Cuddalore (4.5 X 10^4 cfu/g),

Table 1: Total bacterial (Log_{10} of CFU/g) population of surface sediments from the inner shelf of Bay of Bengal, Chennai

T	D(m)	Total bacterial population						
		2006-07		2007-08		2008-09		
		PRM	POM	PRM	POM	PRM	MON	POM
ET	Shore	6.0±0.12[a]	6.1±0.12[a]	6.0±0.12[a]	6.1±0.12[a]	6.1±0.12[a]	6.1±0.12[a]	6.1±0.12[a]
	4m	5.9±0.24[a]	5.9±0.24[a]	5.9±0.24[a]	5.9±0.24[a]	5.9±0.23[a]	5.9±0.24[a]	5.9±0.24[a]
	9m	5.3±0.11[b]	5.3±0.11[b]	5.3±0.11[b]	5.4±0.11[b]	5.3±0.11[b]	5.4±0.11[b]	5.4±0.11[b]
	29m	5.0±0.20[b]	5.1±0.21[b]	5.1±0.20[b]	5.1±0.20[c]	4.8±0.19[c]	5.0±0.20[c]	5.1±0.20[c]
	62m	5.0±0.10[b]	5.0±0.10[b]	5.0±0.10[b]	4.9±0.10[c]	4.8±0.10[c]	4.8±0.10[c]	4.9±0.10[c]
FHT	Shore	6.1±0.24[a]	6.0±0.24[a]	6.1±0.24[a]	6.1±0.25[a]	6.0±0.24[a]	6.1±0.24[a]	6.2±0.25[a]
	5m	5.9±0.12[a,b]	5.9±0.12[a,b]	6.0±0.12[a]	6.0±0.12[a]	5.9±0.12[a]	5.9±0.12[a]	6.0±0.12[a]
	13m	5.7±0.23[b]	5.8±0.23[b]	5.8±0.23[b]	5.8±0.23[b]	5.3±0.21[b]	5.6±0.22[b]	5.8±0.23[b]
	31m	5.3±0.11[c]	5.5±0.11[c]	5.3±0.11[c]	5.0±0.10[c]	5.2±0.10[b,c]	5.0±0.10[c]	5.4±0.11[c]
	60m	5.2±0.21[c]	5.3±0.21[d]	5.3±0.21[c]	5.3±0.21[d]	5.1±0.20[c]	5.0±0.20[c]	5.2±0.21[c]
CHT	Shore	5.9±0.12[a]	6.2±0.12[a]	6.0±0.12[a]	6.1±0.12[a]	6.0±0.12[a]	6.1±0.12[a]	6.2±0.12[a]
	5m	5.9±0.24[a]	5.9±0.24[b]	5.8±0.23[a]	5.9±0.24[a]	5.8±0.23[a]	5.7±0.22[b]	5.8±0.22[b]
	17m	5.8±0.12[a]	5.7±0.11[b]	5.4±0.11[b]	5.4±0.11[b]	5.3±0.11[b]	5.5±0.11[b]	5.6±0.12[b]
	30m	5.4±0.21[b]	5.2±0.21[c]	5.0±0.20[c]	5.1±0.20[c]	5.0±0.20[c]	5.0±0.20[c]	5.3±0.21[c]
	64m	5.1±0.10[c]	4.9±0.10[d]	4.9±0.10[d]	4.9±0.10[c]	4.8±0.10[d]	4.6±0.09[d]	4.8±0.10[d]
CRT	Shore	5.9±0.24[a]	5.9±0.24[a]	5.8±0.23[a]	5.9±0.24[a]	5.8±0.23[a]	5.9±0.24[a]	6.0±0.24[a]
	8m	5.8±0.12[a]	5.4±0.11[b]	5.2±0.10[b]	5.3±0.11[b]	5.2±0.10[b]	5.3±0.11[b]	5.4±0.11[b]
	19m	5.6±0.22[b]	5.0±0.20[c]	4.9±0.20[c]	5.0±0.20[c]	4.8±0.19[c]	4.9±0.20[c]	5.0±0.20[c]
	34m	5.4±0.11[c]	4.8±0.10[d]	4.8±0.10[c]	4.8±0.10[d]	4.8±0.10[c]	4.5±0.09[d]	4.8±0.10[d]
	72m	5.1±0.20[d]	4.7±0.19[d]	4.6±0.18[d]	4.7±0.19[d]	4.5±0.18[d]	4.5±0.18[d]	4.7±0.19[d]
ART	Shore	5.7±0.11[a]	5.9±0.12[a]	5.9±0.12[a]	5.9±0.12[a]	6.1±0.12[a]	5.9±0.12[a]	5.9±0.12[a]
	6m	5.5±0.23[b]	5.4±0.22[b]	5.5±0.22[b]	5.6±0.22[b]	5.9±0.22[a]	5.5±0.22[b]	5.7±0.23[a]
	18m	5.4±0.11[b]	5.3±0.11[b,c]	5.1±0.10[c]	5.3±0.11[c]	5.6±0.12[b]	5.3±0.11[b]	5.4±0.11[b]
	20m	5.2±0.21[c]	5.2±0.21[c]	5.0±0.20[c]	5.0±0.20[d]	5.0±0.20[c]	5.0±0.20[c]	5.1±0.20[c]
	45m	5.0±0.10[d]	5.0±0.10[d]	4.8±0.10[d]	4.9±0.10[d]	4.8±0.10[c,d]	4.8±0.10[d]	5.0±0.10[c]
	65m	4.6±0.18[e]	4.9±0.20[d]	4.6±0.18[d]	4.7±0.19[e]	4.6±0.18[d]	4.7±0.19[d]	4.8±0.19[d]
AVG		5.5	5.4	5.4	5.4	5.3	5.3	5.4
MAX		6.1±0.24	6.2±0.12	6.1±0.24	6.1±0.25	6.1±0.12	6.1±0.24	6.2±0.12
MIN		4.6±0.18	4.7±0.19	4.6±0.18	4.7±0.19	4.5±0.18	4.5±0.09	4.7±0.19

T- Traverses; Values are mean of three replicates with SD. Values in a table with same letter are not statistically significant ($p < 0.05$) according to the Duncan's multiple range test.

Cheyyur (4.2 X 10^4 cfu/g), Chennai (4.6 X 10^4 cfu/g), Tammenapatanam (4.4 X 10^4 cfu/g), Singarayakonda (4.7 X 10^4 cfu/g), Divipoint (4 X 10^4 cfu/g), Kakinada (5.5 X 10^4 cfu/g), Bhemuli (5.2 X 10^4 cfu/g), Barua (5.3 X 10^4 cfu/g) and Paradweep (5.1 X 10^4 cfu/g) recorded by Das *et al*., (2007) was also in support of present findings. Further they have obtained *Pseudomonas* sp., *Bacillus* sp., *Vibrio* sp., *Alcaligenes* sp., *Micrococcus* sp., *Corynebacterium* sp. and *Flavobacterium* sp., as the common organism which are also strongly supporting the present results. Cavallo *et al*., (1999) studied the sediment samples of Mar Piccolo of Taranto, Ionian Sea, Italy and reported the total bacterial population in autumn season mean 3.8 X 10^4 CFU/g sediment, whereas in summer season the mean value was 5.9 X 10^5 CFU/g sediment and strains isolated were *Aeromonas* sp., *Photobacterium* sp., *Moraxella* sp., *Alcaligenes* sp., *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Flexibacter* sp., *Moraxella* sp. and *Xanthomonas* sp.

Density of copper resistant bacteria (CuRB) in surface sediments

Cultivable heterotrophic bacterial communities represent only a small fraction of taxa present in the environment; nevertheless, some authors consider the fraction of the cultivable bacteria to be a useful indicator for measuring shifts on bacterial communities (Stephen *et al*., 1999). Several authors consider estimating the number of cultivable heavy metal-tolerant bacteria more effective in order to establish the toxic effects of heavy metals instead of the number of total cultivable bacteria (Doelman *et al.*, 1994; Huysman *et al*., 1994; Viti and Giovannetti, 2001). The copper resistant bacteria population in the surface sediment samples collected during the study period was in the range of 3.3 to 5.0 log10 CFU/g with an average of 4.4 log10 CFU/g of sediment (Table 2). In the present study, maximum numbers of CuRB populations available during three seasons of three consecutive years were scored during PRM season when compared to MON and POM seasons and this observation was supported by a previous study on metal resistant population on Uppanar estuary in Tamil Nadu, India (Karthikeyan *et al*., 2007). Sathyamurthy *et al*., (1990) isolated organisms from Pichavaram mangroves and reported that the higher metal resistant population was attributed to industrial discharge and terrigenous materials through land runoff.

Table 2: Copper resistant bacteria (Log_{10} of CFU/g) population of surface sediments from the inner shelf of Bay of Bengal, Chennai

T	D(m)	Copper resistant bacterial population						
		2006-07		2007-08		2008-09		
		PRM	POM	PRM	POM	PRM	MON	POM
ET	Shore	4.7±0.11[a]	4.7±0.11[a]	4.7±0.11[a]	4.7±0.11[a]	4.6±0.11[a]	4.7±0.1[a]	4.7±0.1[a]
	4m	4.7±0.23[a]	4.6±0.22[a]	4.4±0.22[ab]	4.5±0.22[a]	4.4±0.22[a]	4.4±0.2[a]	4.6±0.2[a]
	9m	4.4±0.11[a]	4.3±0.11[b]	4.3±0.11[bc]	4.2±0.10[b]	4.3±0.11[a]	4.1±0.1[b]	4.2±0.1[b]
	29m	4.1±0.20[b]	4.0±0.20[c]	4.0±0.20[c]	4.1±0.20[b]	3.9±0.10[b]	4.0±0.1[b]	4.1±0.2[b]
	62m	3.9±0.10[b]	3.8±0.10[c]	3.6±0.09[d]	3.6±0.09[c]	3.5±0.18[c]	3.5±0.2[c]	3.7±0.1[c]
FHT	Shore	5.0±0.23[a]	4.8±0.23[a]	4.7±0.23[a]	4.7±0.23[a]	4.7±0.11[a]	4.7±0.1[a]	4.8±0.2[a]
	5m	4.8±0.12[ab]	4.7±0.12[a]	4.7±0.11[a]	4.7±0.11[a]	4.7±0.11[a]	4.6±0.1[a]	4.7±0.1[a]
	13m	4.6±0.22[b]	4.4±0.11[b]	4.6±0.22[a]	4.5±0.22[b]	4.5±0.22[b]	4.3±0.2[b]	4.4±0.2[b]
	31m	4.3±0.11[c]	4.1±0.20[c]	4.4±0.11[b]	4.3±0.11[c]	4.3±0.11[c]	4.0±0.1[c]	4.1±0.1[c]
	60m	4.3±0.21[c]	3.7±0.09[d]	4.3±0.21[b]	4.0±0.20[d]	4.3±0.11[c]	3.6±0.1[d]	3.8±0.2[d]
CHT	Shore	4.9±0.12[a]	4.9±0.24[a]	4.9±0.12[a]	4.7±0.11[a]	4.8±0.23[a]	4.9±0.2[a]	5.0±0.1[a]
	5m	4.8±0.23[a]	4.8±0.12[a]	4.9±0.24[a]	4.5±0.22[a]	4.8±0.12[a]	4.9±0.1[a]	4.9±0.2[a]
	17m	4.5±0.11[b]	4.2±0.21[b]	4.6±0.11[b]	4.2±0.10[b]	4.5±0.11[b]	4.7±0.1[b]	4.3±0.1[b]
	30m	4.3±0.21[b]	3.6±0.09[c]	4.4±0.22[b]	4.1±0.20[b]	4.3±0.21[bc]	4.6±0.2[b]	3.9±0.2[c]
	64m	4.0±0.10[c]	3.3±0.09[d]	4.1±0.10[c]	3.6±0.09[c]	4.1±0.10[c]	4.1±0.1[c]	3.8±0.1[c]
CRT	Shore	4.9±0.23[a]	4.9±0.24[a]	4.9±0.24[a]	4.9±0.24[a]	4.8±0.12[a]	4.9±0.1[a]	4.9±0.2[a]
	8m	4.6±0.11[b]	4.6±0.11[b]	4.7±0.11[a]	4.7±0.11[a]	4.7±0.23[a]	4.6±0.2[b]	4.7±0.1[a]
	19m	4.4±0.22[bc]	4.3±0.21[c]	4.5±0.22[b]	4.4±0.22[b]	4.5±0.11b	4.3±0.1[c]	4.4±0.2[b]
	34m	4.2±0.10[cd]	4.0±0.10[d]	4.4±0.11[b]	4.3±0.11[b]	4.3±0.11[bc]	4.1±0.1[c]	4.3±0.1[b]
	72m	4.0±0.20[d]	3.7±0.19[e]	4.0±0.20[c]	4.0±0.20[c]	4.1±0.21[c]	3.8±0.2[d]	4.0±0.2[c]
ART	Shore	4.7±0.10[a]	4.7±0.11[a]	4.5±0.11[a]	4.7±0.11[a]	4.6±0.11[a]	4.7±0.1[a]	4.8±0.1[a]
	6m	4.1±0.23[b]	4.5±0.11[ab]	4.4±0.22[ab]	4.6±0.22[a]	4.4±0.11[b]	4.6±0.1[ab]	4.7±0.2[ab]
	18m	4.1±0.10[b]	4.3±0.21[b]	4.3±0.11[bc]	4.4±0.11[b]	4.3±0.21[b]	4.5±0.2[b]	4.6±0.1[b]
	20m	4.0±0.20[b]	4.0±0.10[c]	4.2±0.21[c]	4.3±0.21[b]	4.1±0.10[c]	4.3±0.1[c]	4.4±0.2[c]
	45m	3.6±0.09[c]	3.5±0.18[d]	4.0±0.10[d]	4.1±0.10[c]	3.8±0.10[d]	4.0±0.1[d]	4.1±0.1[d]
	65m	3.5±0.18[c]	-	3.8±0.19[e]	3.8±0.19[d]	3.8±0.19[d]	3.6±0.2[e]	3.8±0.2[e]
AVG		4.4	4.3	4.4	4.3	4.4	4.3	4.4
MAX		5.0±0.12	4.9±0.24	4.9±0.24	4.9±0.24	4.8±0.12	4.9±0.2	5.0±0.1
MIN		3.5±0.18	3.3±0.09	3.6±0.09	3.6±0.09	3.5±0.18	3.5±0.2	3.7±0.1

T- Traverses; Values are mean of three replicates with SD. Values in a table with same letter are not statistically significant ($p < 0.05$) according to the Duncan's multiple range test.

Table 3: Total number of copper resistant bacteria isolates scored in the study area

T	Depth (m)	2006-07 PRM	2006-07 POM	2007-08 PRM	2007-08 POM	2007-08 PRM	2007-08 MON	2007-08 POM	T. wise	Near shore	Inner shelf
ET	Shore	2	2	4	2	3	2	2	45	33	12
	4m	2	1	2	2	5	2	2			
	9m	1	1	1	1	2	1	1			
	29m	1	0	1	1	1	0	0			
	62m	0	0	0	0	0	0	0			
FHT	Shore	2	2	3	2	2	2	2	41	29	12
	5m	2	2	2	2	2	2	2			
	13m	1	1	2	1	1	1	1			
	31m	1	0	1	0	1	1	0			
	60m	0	0	0	0	0	0	0			
CHT	Shore	2	2	3	2	2	2	2	39	28	11
	5m	1	2	2	2	2	2	2			
	17m	1	1	1	2	1	1	1			
	30m	1	0	1	1	0	0	0			
	64m	0	0	0	0	0	0	0			
CRT	Shore	2	2	3	2	2	2	2	40	29	11
	8m	2	2	2	2	2	2	2			
	19m	2	1	1	2	1	1	1			
	34m	1	0	0	1	0	0	0			
	72m	0	0	0	0	0	0	0			
ART	Shore	2	2	3	2	1	2	2	50	28	22
	6m	2	2	3	2	1	2	2			
	18m	1	2	2	2	0	2	2			
	20m	1	1	1	1	1	2	1			
	45m	1	0	0	0	0	1	0			
	65m	0	0	0	0	0	1	0			
Season wise		31	26	38	32	30	31	27			
Year wise		57		70		88					
Total		215									

T; Traverses, m; Meters

The population of heavy metal resistant bacteria was almost similar during the three consecutive years with slight changes. Highest CuRB populations were recorded in sediment samples of harbor regions when compared to the river mouth traverses like ART, CRT and ET (Korttalaiyar River), which scored lesser number of metal resistant populations, the samples taken at shallow depth scored higher CuRB population than the samples of deeper. Similar condition was reported in harbour region of Mar Piccolo of Taranto (Ionian Sea, Italy) (Cavallo *et al*., 1999) and in Eastern Harbour of Alexandria, Egypt (Gouda, 2006). The samples collected at less than 10 m depths were rich in heavy metal resistant populations. Viable bacterial populations in Beaufort Sea sediments were higher in nearshore areas than offshore areas (Kaneko *et al*., 1976). The association of microorganisms with sediment particles is one of the primary complicating factors in assessing microbial fate in aquatic systems. Majority of microbes in aquatic systems are associated with sediments and that these associations influence their survival and transport characteristics (Jamieson *et al.*, 2005; Gouda, 2006; Karthikeyan *et al.*, 2007).

Isolation and identification of copper resistant bacteria

Total number of CuRB isolates

Totally 215 Cu^{2+} resistant bacteria isolates were randomly scored based on morphological differences. During 2006-07, 57 Cu^{2+} resistant isolates were scored of which 31 were obtained from the samples of PRM and 26 were from the samples of POM. Among the 70 isolates scored in the year 2007-08, 38 were from the PRM samples and 32 from the samples of POM. Similarly, during 2008-09, a total of 88 Cu^{2+} resistant bacteria isolates were scored (30-PRM; 31-MON; 27-POM). The isolated CuRB isolates exhibited more numbers in near shore environment when compare to the inner shelf of the study area (Table 3). Wollast, (1991) reported that the coastal and shelf sediments play a significant role in the demineralization of organic matter which supports the growth of microbes. Horizontal and vertical distribution of bacterial populations in sediments is influenced by various factors, such as the physicochemical nature of sediments and the presence of high organic matter concentrations. In aquatic ecosystems, the flux of organic matter to the bottom sediments depends on primary productivity at the ocean surface and on water depth. One clear indication from this study is the observation of steady increase in metal resistant population during the study period, since the heavy metal resistant bacteria population was higher during 2008-09 when compared to 2006-07 and 2007-08.

Identification of CuRB strains isolated based on morphological and biochemical tests

Phenotypic and biochemical characters of the representative heavy metal resistant bacteria isolates are presented in Table 4. Among the copper resistance bacterial isolates scored during the three year study period. *Vibrio* spp. (44) was the most dominant genus recorded from all traverses of the study area. The second dominant genus was *Halomonas* spp. (23) and *Bacillus* spp. (22), which was also isolated from all traverses. The fourth frequently available genus was *Marinobacter* spp. (20), which was followed by *Micrococcus* spp. (19) and *Flavobacterium* spp. (16) and all of these were isolated from all the traverses of sediment samples; whereas *Alteromonas* spp. (14), *Alcaligenes* spp. (14), *Pseudomonas* spp. (12), *Marinomonas* spp. (9) and *Acinetobacter* spp. (8) were obtained from surface sediment samples. About 14 unidentified CuRB strains were also isolated the sediment samples in the study area (Table 5). Some bacterial species have been reported to resist Cu^{2+} even without any prior exposure (Cooksey, 1993). Alavandi, (1989) observed the abundant presence of *Vibrio* sp. in coastal area of Cochin. Recently Parvathi *et al*., (2009) reported *Bacillus* strains as dominant group in the coastal environment of Cochin, India. Promod and Dhevendaran, (1987) and DeSouza *et al.*, (2000) reported *Bacillus* sp. as the dominant group in the inshore areas of west Coast. Every group of the metal resistant isolates of the present study exhibited various levels of resistance against a wide spectrum of heavy metal ions and this observation has been very well recognized by many workers (Yilmaz, 2003; Kamalakannan *et al*., 2006; Adarsh *et al.*, 2007; Roy and Nair, 2007; Abskharon *et al.*, 2008; Altug and Balkis, 2009). Copper-resistance determinants were found in various *Pseudomonas* spp. (Lin and Olson, 1995; Vargas *et al.*, 1995) and in *Xanthomonas campestris* (Lee *et al*., 1994).

Gram-negative isolates were found to be dominant when compared to Gram-positive ones in the study area (Table 5). This observation was in agreement with Moriarty and Hayward, (1982) who reported that the majority of bacteria in marine environments are Gram-negative. Tolerance to heavy metals was also reported to be well pronounced in Gram-negative bacteria (Nair *et al*., 1993; Duxbury, 1986). The majority of the isolates belong to the genera *Pseudomonas* sp., *Vibrio* sp. and *Flavobacterium* sp. (Stolp, 1988).

MIC for CuRB in the study area

After initial screening (0.3 mM $CuSO_4$) CuRB isolates were transferred to media containing subsequent higher concentrations of copper viz., 1.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 mM. The bacterial isolates were able resist $CuSO_4$ to as high as 25.0 mM concentration (Table 6). Out of 215 CuRB isolates selected from surface sediments, 102 were able to grow in 1.0 mM, 45 isolates in 5.0 mM, 23 isolates in 10.0 mM, 14 isolates in 15.0 mM, 6 isolates in 20.0 mM and 1 isolate in 25.0 mM of $CuSO_4$. Most of the isolates that exhibited higher MIC were found to be isolated from river mouth region of the study area. Among the 215 CuRB isolates collected from the study area 5 exhibited resistance upto 20.0 mM and 1 isolate upto 25.0 mM. Vargas *et al.*, (1995) isolated copper resistant *Pseudomonas* sp. which resists Cu^{2+} at 7.8-31.4 mM from water and soil collected from three heavy-metal polluted zones in Central Mexico. Coral *et al.*, (2005) reported *Enterobactor* sp. with a maximum resistance to Cu^{2+} at 25.0 mM. Acosta *et al.*, (2005) reported a strain of *Paenibacillus polymixa* with minimum inhibitory concentration of 3.5 mM of Cu^{2+}.

Table 4: Phenotypic and Biochemical characteristics of metal resistant bacteria isolates

Isolates	Color of the Colony	Gram reaction	Shape	Spore forming	Motility	Catalase	Oxidase	Indole	MR	VP	Citrate utilization	Nitrate reduction	TSI
***Acinetobacter* spp.**	Creamy white	-	rods	-	-	+	-	-	-	-	+	-	±
***Alcaligenes* spp.**	White	-	rods	-	+	+	+	-	-	-	+	-	Fermentation of lactose and sucrose
***Alteromonas* spp.**	Creamy white	-	rods	-	+	+	+	-	+	±	-	-	Fermentation of lactose and sucrose
***Bacillus* spp.**	Creamy white	+	Straight rods, arranged in pairs or chains	+	+	+	+	-	+	±	-	±	±
***Flavobacterium* spp.**	Yellowish	-	rods	-	-	+	+	+	-	-	-	-	Fermentation of lactose and sucrose
***Halomonas* spp.,**	Creamy white	-	rods	-	+	+	+	±	-	-	-	-	Fermentation of lactose and sucrose
***Marinobacter* spp.**	Creamy white	-	rods	-	+	+	+	-	-	-	-	+	±
***Marinomonas* spp.**	Creamy white	-	rods	-	+	-	+	-	-	-	+	-	Fermentation of lactose and sucrose
***Micrococcus* spp.**	Yellowish	+	Cocci	-	+	+	+	-	-	-	-	+	±
***Pseudomonas* spp.**	Yellowish white	-	rods	-	+	+	+	-	-	-	+	±	±
***Vibrio* spp.**	Creamy white	-	rods	-	+	+	+	±	-	-	±	+	Fermentation of lactose and sucrose
Unidentified	±	±	±	±	±	±	±	±	±	±	±	±	±

MR- Methyl Red; VP- Voges Proskauer; TSI- Triple Sugar Iron; +- Positive; - - Negative; ± - Variables

Table 5: Total number of isolated CuRB strains in the study area

Strains	2006-07		2007-08		2008-09			ST
	PRM	POM	PRM	POM	PRM	MON	POM	
Gram Negative								
***Acinetobacter* spp.**	1	1	2	1	1	1	1	8
***Alcaligenes* spp.**	2	2	2	2	2	2	2	14
***Alteromonas* spp.**	2	2	2	2	2	2	2	14
***Flavobacterium* spp.**	3	1	3	3	2	3	1	16
***Halomonas* spp.,**	3	3	5	3	3	3	3	23
***Marinobacter* spp.**	2	4	4	3	2	2	3	20
***Marinomonas* spp.**	1	1	3	1	1	1	1	9
***Pseudomonas* spp.**	2	1	2	2	2	2	1	12
***Vibrio* spp.**	7	5	7	7	7	7	4	44
	23	20	30	24	22	23	18	160
Gram Positive								
***Bacillus* spp.**	3	3	3	3	3	3	4	22
***Micrococcus* spp.**	3	2	3	3	3	3	2	19
Unidentified	2	1	2	2	2	2	3	14
	8	6	8	8	8	8	9	55
	31	26	38	32	30	31	27	
	57		70		88			
Total	215							

ST; Species wise Total

Chisholm *et al*., (1998) reported 15 isolates of *Thiobacillus ferrooxidans* recovered from the tailings fields of a Cu-Ni mine that showed inhibitory concentrations of Cu^{2+} and Ni^{2+} with a range of 80.0 mM to more than 320.0 mM to both the metals.

Multiple metal ions resistance in CuRB isolates

The CuRB isolates which showed growth in medium with above 20.0 mM of $CuSO_4$ were selected for further studies. The 6 isolates thus selected were named such as VKRKCu1 to VKRKCu4 and VKRKCu8 & VKRKCu9 were screened for multiple metal resistances. They showed resistance to various other metal ions at different levels (Table 7). Four of the 6 selected Cu^{2+} resistant isolates showed resistant to Ni^{2+} upto 15.0 mM, for all the selected isolates are resisted cadmium upto 6.0 mM, 4 showed resistance to Co^{3+} at 8.0 mM, 3 resisted lead upto 8.0 mM, 1 upto 8.0 mM Cr^{6+} and Zn^{2+}, 2 upto 8.0 mM Fe^{3+} and Mn^{2+} and no growth in Hg^{2+} at 1.5 mM. The isolation of copper resistant isolates, which could tolerate such high levels of metal ions, has provided an opportunity to infer that these organisms were under constant metal stress exhibited in the study area due to anthropogenic input. The use of bacteria for rehabilitation of polluted environments may provide an ecologically sound method for abatement of pollution and a natural solution for recovery of contaminated sediment (Gupta and Ali, 2004). Thus, this study provides convincing evidence for a prolonged modification of the indigenous bacterial community caused by transient exposure to Cu^{2+}. Most of the strains were able to grow at high concentrations of Cd^{2+}, Co^{3+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cr^{6+}, Fe^{3+}, Hg^{2+} and Mn^{2+}, which might be important for the capacity of these bacteria to survive in different sources of pollution with elevated level of heavy metal ions. Multiple tolerances occur only to toxic compounds that have similar mechanisms underlying their toxicity. Since heavy metals are all similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria.

The results of the present study clearly showed the increase in the development of metal resistance among the bacterial population during the study period. Further, there was also an uptrend in the concentration level of resistant development in the bacterial populations. For example, the highest concentration for Cu^{2+} resistance recorded during 2006-07 was 20.0 mM which was enhanced to 25.0 mM during the 2007-08 and 2008-09. High number of metal

Table 6: Minimum inhibitory concentration (MIC) for CuRB isolates in the study area

Seasons	T	Cu^{2+} concentration in mM							
		0.3*	1	5	10	15	20	25	30
2006-07	ET	6	4	2	2				
PRM	FHT	6	3	1	1				
	CHT	5	2	1					
	CRT	7	3	1					
	ART	7	4	2	2	2			
2006-07	ET	4	2	1					
POM	FHT	5	2						
	CHT	5	2						
	CRT	5	3	1					
	ART	7	3	2					
2007-08	ET	8	4	2	2	1			
PRM	FHT	8	3	1					
	CHT	7	2	1					
	CRT	6	3	1					
	ART	9	5	3	2	2	1		
2007-08	ET	6	3	1					
POM	FHT	5	3	1					
	CHT	7	3	2					
	CRT	7	3	1	1				
	ART	7	3						
2008-09	ET	11	5	3	2	1			
PRM	FHT	6	3	1					
	CHT	5	1	1					
	CRT	5	3	2	2	2	1	1	
	ART	3	2	2	1	1	1		
2008-09	ET	5	2	2	1	1	1		
MON	FHT	6	4	1	1				
	CHT	5	2						
	CRT	5	3	1					
	ART	10	4	2	2	1			
2008-09	ET	5	2						
POM	FHT	5	2						
	CHT	5	3	1					
	CRT	5	2	2	2	1	1		
	ART	7	4	3	2	2	1		
Total		215	102	45	23	14	6	1	Nil

*****-*Initially screened concentration; T- Traverses.*

Table 7: Multiple metal resistance spectrum of CuRB isolates in the study area (Conc. in mM)

Isolates code	MIC	Ni			Cd			Co			Pb			Hg			Cr			Fe			Mn			Zn		
		15	20	25	6	8	10	6	8	10	6	8	10	1.5	2	2.5	6	8	10	6	8	10	6	8	10	6	8	10
VKRKCu1	20	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	-	-	-
VKRKCu2	20	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-
VKRKCu3	25	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-	+	+	-	+	+	-	+	-	-	+	-	-
VKRKCu4	20	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	-	-
VKRKCu8	20	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	+	-	-
VKRKCu9	20	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	-

resistance bacterial isolates were scored in ART and was followed by ET > FHT > CRT > CHT. Adyar river bank is highly populated and situated with small and medium scale industries which are the source of metal pollution. Cooum River is running thro thickly populated areas of Chennai. In addition to the human settlement along the banks may of the small industries are the sources of metal pollution in this zone. This increase might be due to the rise in industrial development and population. Furthermore, for many years, heavy wastes from nearby streams have affected this region. In urban areas, runoff from yards, sewage overflows, and sewage discharges during rain events may increase the total number of microbes at the beach (Sampson *et al*., 2006). The maximum metal accumulation was attributed to higher heavy metal discharge mainly through in anthropogenic inputs, industrial effluent or land run-off (Karthikeyan *et al*., 2007). An increase in the resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is due to the growth primarily of the resistant bacteria (Barkay and Olson, 1986; Muller *et al*., 2001b; Rasmussen and Sorensen, 2001). The frequency of tolerant microorganisms may increase with an increase in toxic metal levels (Huysman *et al*., 1994; Kunito *et al*., 1997). This can lead to a decrease in species diversity and therefore a shift in microbiota composition (Pennanen *et al*., 1996). Shakibaie and Harati, (2004) suggested that the incidence of a high metal resistant population resulted from increasing environmental pollution.

In conclusion, the diversity of culturable metal resistant population in the study area was found to be significantly higher when compared to previous studies. But the diversity was definitely smaller than that could be found in less contaminated sites. The physiological stress caused by the toxic effect of metal ions lead to the selection of less diverse communities comprising of metal-resistant populations (Sandaa *et al*., 1999; Feris *et al*., 2003). General suppression of metabolic activity of organisms could also be a cause for less diversity (Komulainen and Mikola, 1995). Thus, the continuous stress of heavy metal ions in the study area is the cause for less diversity. Hence, there is an urgent need for controlling the input of the metal ions in the coastal zone of Chennai. Effective legislations and continuous monitoring may be ensured to at least sustain the present level of pollution that may be slowly cleaned by the activity of indigenous microbial system.

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