

## 2.1. INTRODUCTION

Comprising <0.5 to 4  $\mu\text{m}$  sized single cells, bacterial communities are of immense importance both for ecosystem function and stability. Most uniquely this prokaryotic life can develop, adapt and survive in the most inhospitable surroundings. Their physiological responses such as viability (Colwell et al., 1985; Oliver et al., 1995; Naganuma, 1996), metabolism, resting stages and death (Amy & Morita, 1983) are caused by certain adverse anthropogenic and some natural processes. As natural assemblages experiencing the 'wilderness' imposed through pollution by anthropogenic expansionism, bacteria adapt quite rapidly and gear up themselves to cope up with adversities, rather successfully, to a very great extent. Monitoring bacterial responses of any type is useful for assessing marine microbial heterotrophy (Kogure et al., 1987) and environmental quality (Liebert & Barkay, 1988; Ramaiah & Chandramohan, 1993; Ramaiah et al., 2002a, 2002b). Thus, for a long time now, many investigations have been using bacterial indicators (e.g., coliform groups) to assess effluent discharges into the coastal/ marine environment. Long-term exposure to heavy metals viz. Zn, Cu and Ni (Bååth et al., 1998) and Hg (Rasmussen & Sørensen, 1998, 2001; Müller et al., 2001a) has been found to alter the microbial community. Observations on occurrence and distribution of native bacteria capable of metal tolerance are of relevance in microbial ecology to understand the extent of metal pollution (Rasmussen & Sørensen, 2001; Müller et al., 2001b) and to realize the potential of such flora in detoxifying toxic substances (Barbieri et al., 1996; Ka et al., 1994; De et al., 2003). Many human activities have negative impact on several biological processes and there is no doubt that these will continue to affect the functioning of highly productive coastal ecosystems. Contamination caused by heavy metals like mercury affects both oceanic and those of continental shelf and coastal regimes, where besides longer residence times, metal concentrations are higher due to the anthropogenic input, transport by river runoff and the proximity to industrial and urban zones

(Nriagu, 1990; Rainbow & Furness, 1990). Widespread mercury contamination of the coastal zones grows alarmingly with time and is already affecting most marine ecosystems in the Caribbean region (Rawlins et al., 1998). Many studies on the biota, sediments and water have reported concentrations far above the levels tolerable by humans (Knight et al., 1997; Moreira & Piveta, 1997; Olivero & Solano, 1998; Bastidas et al., 1999a, 1999b).

Leaching naturally (Gerlach, 1981; Anon, 1998) and added anthropogenically, mercury (Hg) keeps accruing in seawater and marine sediments. Passive uptake of Hg and its compounds by many osmotrophs including bacteria leads to their modification and, in changed forms, they move up the food chain, get bio-magnified and reach human beings where they produce chronic and/or acute ailments (Förstner & Wittmann 1979). In addition to natural processes of bedrock leaching, Hg and its compounds enter aquatic bodies by washings from soils and atmosphere (Mason et al., 1994), effluents from chlor-alkali production units (Colwell et al., 1985; Anon, 1999; Nahida et al., 2000) chemical laboratories, instrument manufacturing, dentistry and fluorescent light bulb breakage (Hütter (1978) and from sewage treatment facilities (Soldano et al., 1975). Our current understanding is that due to very slow biogeochemical remobilization, toxic heavy metals (e.g., Hg, Cd, Cu, Pb) and metalloids (e.g., As) forming complexes with organic components of marine sediments (Förstner & Wittmann, 1979; Gerlach, 1981; Barkay, 1987; Gilmour et al., 1992) tend to accumulate leading ultimately to deleterious situations to marine life and in turn, human beings. Thus, environmental effects of metal pollution are all encompassing (Baldi et al., 1989).

Worldwide many areas are mercury polluted and present a threat to people and environment (Fukuda et al., 1999; Horvat et al., 1999). As a consequence, mercury-resistance is often seen to

be associated with the natural flora (Pahan et al., 1990; Canstein, 1999; Macalady, 2000; Müller et al., 2001b; Ramaiah & De, 2003). Recently, stringent legislation in the US and European countries have brought down the anthropogenic input and, consequently, the global mercury estimate is on the decrease (Slemr, 2003). The toxicology and environmental behaviour of mercury is quite complex, since the toxicity, mobility and bioaccumulation of mercury depend on its chemical form (D'Itri, 1990). Mercury ore, cinnabar (HgS), poses a limited direct threat because of its low solubility (Morel & Hering, 1993). However, under toxic conditions -as existing in surficial sediments, soils and in most surface waters – HgS can be converted to dissolved divalent mercury ( $\text{Hg}^{2+}$ ), elemental mercury ( $\text{Hg}^0$ ) and methyl mercury ( $\text{CH}_3\text{Hg}^+$ ; Klaassen et al., 1986) latter being the most toxic, which created havoc in the early 1960s in Japan resulting in the Minamata disease. Humans are exposed to methyl mercury principally through the consumption of marine fish and fish products, yet research on Hg transformations and mobility in the marine environment is sparse. Environmental studies have focused on terrestrial regions, especially freshwater ecosystems. In contrast, attention to estuaries and adjacent coastal waters that are major repositories for natural and river borne/watershed derived Hg species is scanty. There is a vital need to increase our knowledge and understanding concerning the biogeochemical cycling of Hg and the impact of anthropogenically related inputs in biologically productive nearshore regions (Fitzgerald et al., 2000).

Mercury-resistant bacteria (MRB) are widely distributed and quite ubiquitous in nature accounting ca. 1-10% of aerobic heterotrophic bacteria (Müller, 2001a). They can be isolated without prior enrichment. However, resistant strains are more abundant in mercury-polluted environments, where up to 50% may grow on nutrient agar media amended with as high as 50  $\mu\text{M}$  (10 ppm)  $\text{Hg}^{2+}$ . Whereas, sensitive strains can at best tolerate ca. 1  $\mu\text{M}$  in the growth media

(Barkay, 2000). The presence of MRB is often correlated with the level of mercury contamination in an environment, although they have been isolated from uncontaminated environments (Osborn et al., 1993).

Although research on mercury resistance and bacteria is into the fifth decade, there have been very few reports on MRB from the marine environments (Garcia et al., 1999). Further, in an open system like oceans, mercury vapour released by resistant bacteria will become part of the local mercury cycle and repollute the environment as has been reported in case of the Amazon river basin (Lacerda & Pfeiffer, 1992). As an integral part of a detailed study on mercury-resistant bacteria (MRB) from marine environment, their abundance and distribution along the Indian coasts were investigated.

## **2.2. MATERIALS AND METHODS:**

Prior to this study for estimating MRB abundance from various locations along the Indian coasts, previous investigations at the National Institute of Oceanography, were routinely examining many indicator and human pathogenic bacteria including MRB since 1993. During the earlier efforts, water and sediment samples were plated on seawater nutrient agar (SWNA) containing 0.5 ppm ( $\approx 2.5\mu\text{M}$ ), 1 and 5 ppm Hg (as  $\text{HgCl}_2$ ). The MRB were not all that abundant prior to and during 1997 either off Chennai, one of the much polluted regions on the east coast or off Positra, a non-polluted region on the West coast. Upon noticing the unusually high counts of MRB during 1999 in SWNA with 5 ppm Hg, various higher concentrations of Hg (i.e., 20, 30, 50, 75 and 100 ppm) were added to well-defined SWNA and the quantification of MRB was carried out for this study.

### **2.2.1. Study sites:**

Various locations sampled for this study are shown in Figures 2.1 and 2.2. Sampling was carried out from polluted (Mumbai, Chennai, Mangalore, Kulai, Padubidri and Ratnagiri), moderately polluted (Malvan, Paradip, Nagpattinam) and non-polluted (Positra, Marmugao and Terekhol) coastal locations. Offshore sampling was carried out during the Bay of Bengal Process Studies (BOBPS) summer monsoon (July-August, 2001) cruise covering two transects, one in the open sea (along 88° E) and another along the eastern coast of India.

The coastal regions off Chennai and Mumbai receive a wide variety of industrial, urban and shipping related effluents and are among the most pollution affected zones along the Indian coast. During the last 15 years or so the coastal waters off Mangalore, Kulai and Padubidri have undergone rapid changes owing to very speedy industrialization, expanded harbor activities and an increasing human settlement (presently from these three places population is >4 million). Varieties of effluents, some after treatment, reach the seas around here and, because of bulk quantities; they affect a large span of coastal water similar to those off Mumbai and Chennai. Both treated and untreated discharges from manufacturing units (e.g., polyvinyl chloride [PVC] pipes and other plastics) and urban effluents from Ratnagiri town (population >1.8 million) reach the coastal waters. The sea off Malvan receives ~0.5 million liters of effluents per day from iron-ore mining activities in addition to offshore oil seepage (Anon., 1997). Although no pollution effect is yet attributable (Anon., 1997), the sea off Terekhol receives ca <0.04 million litres of iron ore mining effluents per day. The coastal zones off Marmugao in the West coast and Gopalpur on the East coast are, to a very large extent, less perturbed. The nearshore intertidal region off Positra has a vast expanse of coral reef with very rich biodiversity. There is least or no ecological perturbation at present.

The study areas from the Bay of Bengal receive a huge amount of freshwater discharges from some major eastflowing Indian rivers. It may be pointed out that the so-called coastal track was not very much within the shelf, but in fairly deep waters (often in water depths exceeding 1000 m) because of the narrow shelf. While the Bay is known for low chlorophyll productivity (Prasannkumar et al 2002), effects of heavy metal pollution on microbial communities are not investigated, in general, from the Bay. Several of these coastal areas have been reported to have higher mercury concentrations (Kaladharan et al., 1999; <http://in.rediff.com/money/2003/nov/04mercury.htm>). Three stations each from offshore track (along 88 °E) and along the coastal track were sampled from surface to 1000 m for quantifying MRB and to realize their percentage in the total viable fractions of heterotrophic bacteria in the water column.

### **2.2.2. Enumeration of MRB:**

During most sampling occasions, water and sediments samples were collected from 9 or 12 stations within 30-35 Km<sup>2</sup> at each sampling location. At least three water samples and one sediment sample were collected along 5, 10 and 15 m depth contours during each collection. During 1993-1997, samples from different locations (Tables 2.1 and 2.2) were plated on to seawater nutrient agar (SWNA with composition l<sup>-1</sup>: peptone [Difco] 5.0g, yeast extract [Difco] 3.0g, agar [Difco] 15g, aged seawater 500 ml and deionized water 500 ml and pH 7.5±0.1) containing 0.5, 1 and 5 ppm Hg. Up to 25 ml water samples and 3 ml supernatants of 10 and/or 100 times diluted sediment samples (lower dilutions plated for clayey sediments) were filtered through 0.22 µm membrane filters and placed on SWNA containing Hg for enumerating MRB. As mentioned earlier, upon noticing very high counts for the first time on all plates with 5 ppm Hg in February 1999 from all samples collected off Marmugao (Goa), we increased the

concentration of Hg to 10, 20, 30, and 50 ppm. Though the CFU from plates with 50 ppm sometimes exceeded those counted previously from SWNA plates with 5 ppm Hg, to be consistent, we decided to use 10 ppm for MRB enumeration reported here. For enumeration of MRB from the samples from the Bay of Bengal, 10-15 ml water sample was filtered through 0.22  $\mu\text{m}$  filters depending on the sampling depth (higher volume of water was used for deeper water). Plates were incubated at room ( $28\pm 2$  °C) or at ship's temperature ( $21\pm 2$  °C) and final counts of CFU taken after 48h. Total plate counts from each sample were also enumerated by plating them on SWNA without added Hg.

### **2.3. RESULTS:**

#### **Enumeration of MRB:**

In 1993, bacteria growing on SWNA amended with 0.5 ppm Hg from water samples collected off Goa ranged from 1.8 to 15.2 cells  $\text{ml}^{-1}$  whereas the range was as high as 2.5 to 10575 cells  $\text{ml}^{-1}$  grown on SWNA amended with 10 ppm Hg (Table 2.1.1). Those counts from sediment samples were in the range of  $4.08 \times 10^4$  to  $1.3 \times 10^7$  cells  $\text{g}^{-1}$  dry weight (Table 2.1.2). MRB counts from water off Mangalore in 1993 ranged from 18.4 to 156.3 cells  $\text{ml}^{-1}$  and those ranged from 27.5 to 960 cells  $\text{ml}^{-1}$  in 2000 (Table 2.2.1) whereas those counts from sediment samples were in the range of 45.63 to  $7690.25 \times 10^4$  cells  $\text{g}^{-1}$  dry weight (Table 2.2.2). In all the three years during 1999 to 2002, the MRB in waters off Gopalpur was always quite high and ranged from 1.4 to 660 cells  $\text{ml}^{-1}$  during 1999, 36 to 120 cells  $\text{ml}^{-1}$  during 2000 and 262.5 to 1712.5 cells  $\text{ml}^{-1}$  in 2002 (Table 2.3). The MRB counts from the sediment samples were in the range of <1% to 97% of the total CFU during 1999 and in 2002 (Figure 2.3), from 5 to >100% (Table 2.3). The MRB in water were always less than 1% of CFU from all the 9 samples from Chennai during 1996 whereas the

range went as high as 100% during 2001 (Table 2.4). During 2001, the MRB in sediment samples off Chennai ranged from  $9.7 \times 10^5$  to  $5.2 \times 10^6$  cells  $\text{gm}^{-1}$  dry weight (Figure 2.4). The counts of MRB off Ratnagiri during 1999-2001 showed high abundance ranging from  $1.4 \times 10^3$  to  $8.9 \times 10^3$  cells  $\text{ml}^{-1}$  during 1999 and from  $1.6 \times 10^2$  to  $1.5 \times 10^4$  cells  $\text{ml}^{-1}$  during 2001 (Table 2.5). These CFU accounted for mean percentages of 1.24% of CFU off Goa and 4.86% off Mangalore (Table 2.6). Furthermore, MRB were none to <3.45% of CFU in 58 samples — i.e., 20 samples from ca. 60 Km Chennai-Kalpakkam stretch off Tamil Nadu coast; 22 from a 120 Km Mangalore-Bhatkal stretch off Karnataka and, 16 samples from 80 Km Talpona -Terekhol off Goa — examined during 1994. In spite of the large volumes of samples examined, there were no colony formations on SWNA with 5 ppm Hg from any of the 214 samples examined until December 1997.

Despite the current pollution levels being low, very insignificant or none off Goa (Marmugao, Terekhol), Nagpattinam and Gopalpur, the MRB growing on SWNA with 10 ppm ( $50 \mu\text{M}$ ) Hg were quite high, always over 13% of the CFU during 1999 (Table 2.7). Apparently, as can be seen from Table 2.7, their percentages have shown increments during 2001 and 2002 from most locations where repeat sampling was possible and done. Off Karwar, MRB in water in March ranged from 13.3 to 366.7  $\text{ml}^{-1}$ ;  $138.3 \pm 158.5$  forming 15.4% [n=4] of the CFU. In sediments it ranged from 1.1 to 1.14  $\text{g}^{-1}$  dry wt;  $1.12 \pm 0.29$  forming 20% [n=4] of the CFU. Off Mumbai, the MRB formed a staggering 96% of water [n=4] and 71.4% of sediment [n=3] CFU during May.

The MRB were present in almost all samples from the coastal as well as oceanic waters. Their occurrence showed an increase in abundance (% of TVC) below 100 m. In case of the two coastal stations located off Orissa ( $19^\circ \text{N}$ ,  $85^\circ \text{E}$ ), and Chennai ( $12^\circ \text{N}$ ,  $82^\circ \text{E}$ ), the MRB percentage increased with depth. The counts (cells  $\text{ml}^{-1}$ ) of MRB ranged from 3.25 to the highest 163.2 forming a maximum of 68% of the CFU at station  $19^\circ \text{N}$ ,  $85^\circ \text{E}$  whereas at  $12^\circ \text{N}$ ,  $82^\circ \text{E}$



the MRB ranged from nil to 613.3 forming more than 92% of the CFU. Similar trend was found at the other coastal stations off at 15° N, 81° E, but the increase in MRB was discernible upto 400 m and then their percentage decreased unlike the other two coastal stations. Interestingly enough, the MRB never exceeded 50% at any depth at the station 15° N, 81° E although the MRB ranged from 0.4 to 139.2 no ml<sup>-1</sup> (Figure 2.6). The oceanic stations also showed similar trend of increased percentage of MRB at deeper waters (Figure 2.7) though the bacterial abundance (general bacteria as well as MRB) were less at the oceanic stations when compared to their density in samples from coastal stations. The MRB maximum (54.4 cells ml<sup>-1</sup>) and minimum (0.9 cells ml<sup>-1</sup>) at the southern most oceanic station 9° N, 88° E were among the least. MRB at the northern most oceanic station (20° N, 88° E) ranged from 2 to 256 cells ml<sup>-1</sup> forming more than 92% of CFU. The other oceanic station was at 15° N, 88° E and had a maximum MRB ranging from 17.86 cells ml<sup>-1</sup> to 294 cells ml<sup>-1</sup> forming over 53% of the total CFU. In terms of their mean percentage concentrations, MRB contributed in the Bay of Bengal over 20% of TVC in the surface (1-10 m), 12% of TVC in 100 m (below mixed layer), 35% at 500 m and 49% at 1000 m.

#### **2.4. DISCUSSION:**

Due to prolonged exposure to mercury and other heavy metal cations/anions, aquatic bacteria can acquire highly specific resistance (Barkay, 1987; Rasmussen & Sørensen, 1998, 2001). Some bacteria can reduce Hg<sup>2+</sup> and most organomercurials to elemental Hg<sup>0</sup>, which volatilizes out of the system due to high vapour pressure (Fitzgerald & Mason, 1997). There is considerable evidence on mercury resistance among common microbial species (Amy & Morita, 1983; Compeau & Bartha, 1984; Colwell et al., 1985; Barkay & Turner, 1997; Barkay, 2000). Some bacteria can convert Hg<sup>2+</sup> to methyl and dimethyl mercury (Gerlach, 1981). Organomercurial

lyase that cleaves the carbon-mercury bonds of certain organomercurials and mercuric reductase that reduces  $\text{Hg}^{2+}$  to volatile mercury helps bacterial cells in detoxifying mercury compounds (Barkay, 1987). The abilities of environmental isolates to tolerate Hg, its various ionic and molecular forms are of greater interest in microbial ecology. From analyzing the available data on mercury concentrations along the Indian coast (Tables 2.6 and 2.7; also see Ramaiah et al., 2002a), it is discernible that increased use of heavy metal applications, in particular of Hg, for industrial and agricultural practices increase in the concentration of this deadly heavy metal in the marine environment. In addition, from wherever the recent data are available, an increasing trend in the concentration of Hg both in water and sediments can be discerned (Kaladharan et al., 1999) including the Indian Ocean region (Sarin, 1991)

Technical publications of NIO during 1990-1999, report Hg in the range of 10-338  $\text{ng g}^{-1}$  dried sediment and 10-285  $\text{ng l}^{-1}$  seawater along the Indian coast. These ranges are higher than the global ocean averages estimated during the 1970s (Gerlach, 1981). Mercury imports to India have more than doubled between 1996 and 2002 from 254 tonnes a year to 531 tonnes annually. Imports of organomercury compound (pesticides, biocides, etc) have jumped 1,500 times - from 0.7 tonnes to 1,312 tonnes during the same period. India has now replaced the US as the biggest consumer of mercury. It consumes 50 per cent of the global production and processes 69% of it. While mapping the "mercury hotspots" in the country, Center for Science and Environment (CSE) found that coastal areas of Mumbai, Kolkata, Cochin, Karwar and Chennai were severely polluted, contaminating the fish stock. In most cases, the contamination level in fishes exceeded the 0.5 ppm total mercury regulation. In west coast, particularly in Mumbai, it was 1.6 times higher than the permissible level. The CSE has reported high levels of mercury in fishes along the coastal areas both in freshwater and saline water studies by the Central Pollution Control

Board and state pollution control boards (<http://in.rediff.com/money/2003/nov/04mercury.htm>).

This increased concentration of Hg in marine regimes ushers in a situation to select microbial assemblages that become capable of high tolerance to Hg. In addition, probably due to their ability to detoxify various mercuric complexes (Gilmour et al., 1992; Müller, 2001a) that perpetually form and undergo ionic changes *in situ*, the numerical abundance of MRB in sediment samples examined during this study was quite high in all locations. In marine environments, mercury resistance may be due to reduction of  $\text{Hg}^{2+}$  to the less toxic and volatile  $\text{Hg}^0$  (Barkay, 1987). This process can, in time, eliminate mercury from polluted environments, resulting in a reduced selection pressure (Barkay et al., 1992). Since the top layers of water and sediment are best aerated and have the highest concentration of easily degradable carbon sources and biomass, it is likely that the energy demanding reduction of mercury is the highest in these regions, resulting in the lowest selection pressure ensuing survival of Hg-sensitive bacterial population (Rasmussen & Sorensen, 1998). This might account for the comparatively lower occurrence of MRB population in the surface and subsurface waters of the BOB whereas the deeper water had higher percentage of MRB. However, as per the current knowledge, the current status of Hg concentration surrounding the Indian subcontinent is quite high and extensive usage of this metal does add up to the atmospheric Hg repository. So, the ocean is also no more a no-pollution zone rather is a reservoir of this metal on a local scale since Hg cycle includes reversible movement of the metal in between sea and atmosphere. Owing to this, the MRB concentration in the BOB was expected to be reasonably high and this study the first of its kind from the BOB in particular and along the Indian coast in general is useful to recognize the high abundances of MRB.

Many previous studies from the European and North American coasts have reported the occurrence of culturable heterotrophic bacteria capable of tolerating ca. 0.5 ppm (2.5  $\mu$ M) Hg from locations affected by a variety of anthropogenic activities (Barkay, 1987; Rasmussen & Sørensen, 1998; Reyes et al., 1999). An increase in the heavy metal resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is ascribed to the growth primarily of mercury-resistant bacteria (Barkay & Olson, 1987; Müller et al., 2001b; Rasmussen & Sørensen, 1998, 2001). Similar to the results obtained during this study prior to 1999, none to 10% of the CFU have been reported to be growing in general nutrient agar media amended with 0.5 ppm Hg. Rasmussen and Sørensen (1998) noticed high levels of self-transmissible Hg resistance plasmids in bacterial communities from a mercury-contaminated site inside the Copenhagen Harbor which had higher abundance of MRB. These authors reported 62% of the isolates from this location to contain plasmids while about 30% of the isolates had plasmids from a non-contaminated site though there were no differences in Shannon-Weaver diversity indices of the isolates from polluted and non-polluted locations. As seen in this study, as many as 25% (21 of 120 isolates) of the Hg sensitive isolates became Hg resistant when grown in very close proximity of MRB (in chapter 4). Although this is not a direct evidence, as Rasmussen and Sørensen (2001) also suggest, it is likely that many species of marine and estuarine bacteria acquire mercury resistance through inter-species horizontal gene transfer.

Silver & Phung (1996) proposed that toxic heavy metal resistance determinants might be preexistent to human activities. In all likelihood, varieties of natural prokaryote assemblages possessing resistance mechanisms would get 'selected' as a result of human pollution (Förstner & Wittmann, 1979) in recent centuries. Such assemblages are of continued relevance in ecosystem stability. It is therefore, of pertinence to take note of adaptive responses by native microflora and

decipher their involvement in biogeochemical cycling of mercury on a global scale. Heavy metal resistant microorganisms do not arise by chance and, that there must be selection factors like environmental contamination by heavy metals (Hideomi et al., 1977). Ecological implications of increased mercury tolerance/resistance observed in this study could mean higher rates of biotransformation of toxic heavy metals; their higher mobilization through marine food web and increased levels of  $\text{Hg}^0$  in the atmosphere. As prokaryotic metabolic pathways dealing with elemental mercury (Smit et al., 1998) or its many inorganic salts generally lead to production of more toxic forms (e.g., alkylmercury), consequences of enhanced atmospheric  $\text{Hg}^0$  could bring about highly undesirable environmental changes. Since  $\text{Hg}^0$  is rapidly oxidized to  $\text{Hg}^{2+}$  by molecular oxygen (Magalhaes & Tubino, 1995) in marine environment, it can lead to formation of a variety of mercuric complexes, all of which are deleterious to life systems. In addition, under anaerobic culture conditions, some bacteria are not harmed by mercury as it is changed directly into nontoxic mercuric sulfide (Macalady et al., 2000). Also, there are recent evidences that under highly anaerobic environments, many transitional metals including Hg form highly soluble anionic polysulfides that are bioavailable (Barkay & Turner, 1997; Barkay, 2000; Winfrey, 1985; Winfrey & Rudd, 1990). However, under non-sulfide, low to moderately oxic conditions, continued accumulation in the sediment would lead to complete elimination of heavy metal sensitive flora and fauna in turn leading to impedance of biological processes in natural habitats subjected to heavy metal contamination. The facts that - a minimum of 13% of CFU and 69% of 120 isolates from routine but well defined SWNA grew on 10 ppm Hg – are strong indications of significant numbers of native bacteria to be already resistant to Hg. In their studies Reyes et al. (1999) found 35-55% of bacteria from the marine environments to be resistant to mercury.

The increasing MRB abundance in the water column does indicate an increase in the Hg concentration in the coastal water off India. At the same time detoxification of mercury by means of volatilization as revealed in the laboratory studies do lead to the speculation that this increase in MRB would influence the Hg<sup>0</sup> repository in the atmosphere and thereby play a major role in controlling the biogeochemical cycle locally. In their studies Fitzgerald et al. (2000) have proposed that Hg flux from coastal waters to atmosphere are likely to be significant locally, regionally and globally. Large quantities of Hg can be remobilized from such waters and microbially mediated recycling provides additional opportunities for methylation and contamination in other aquatic settings both terrestrial and marine. In this study, it has been found that the increase in MRB has been all over irrespective (including some of the offshore locations in the BOB) of the pollution status of the locations. Moreover, low productivity in the BOB during inter monsoon (Prasannakumar et al., 2002) accounts for the relatively low percentage of MRB in the top layers of water column. It is however accepted that the mercury in the food chain is potentially hazardous for the environment and human health (Pilgrim et al., 2000). Long-range atmospheric transport or through marine currents to over thousands of kilometers in only a few months (Rasmussen & Sørensen, 1998) lead to contaminating pristine marine zones.

Long-term monitoring and analyses of available data as done during this study are useful to realize that already a substantial fraction of CFU and, diverse groups of marine bacteria, are capable of high tolerance for mercury and that they are rapidly undergoing physiological and/or genetic modifications for coping up with changing marine ecosystems. High counts of MRB from every examined location in almost every sample after 1999 are strong proofs of the possibility of natural selection of mutants with an advantage to deal with adversities. Such environmental

isolates are potentially useful in heavy metal pollution bioremediation and might reveal new facts about the mercury-resistance mechanisms.

**Table 2.1.1.** Total viable counts (TVC), counts of MRB in water (no. ml<sup>-1</sup>) from Goa, west coast, India during 1993-2001 and their percent in the TVC on seawater nutrient agar amended with <5ppm Hg prior to 1999 and with 10 ppm mercury from 1999 onwards

Year	1993			1999			2000			2001		
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
no. of water samples	6	38	10	20	20	20	20	20	20	20	20	20
	15.2	50	30.4	1150	4945	23.26	5020	17386.67	28.87	3765	2313.33	162.75
	3.4	203	1.67	1837.78	2120	86.69	65	180	36.11	330	220	150
	8.1	57.5	14.09	175.56	1005	17.47	502.5	340	147.79	720	713.33	100.93
	2.6	17	15.29	391.11	875	44.69	110	53.33	206.25	335	506.67	66.12
	1.8	68.5	2.63	70	240	29.17	340	233.33	145.71	1380	2320	59.48
	8.6	82.5	10.42	5555.56	6080	91.37	32.5	26.67	121.87	30	6.67	450
				630	2110	29.86	67.5	280	24.11	502.5	500	100.5
				203.33	525	38.73	275	286.67	95.93	495	493.33	100.34
				275.56	1350	20.41	610	580	105.17	2160	4533.33	47.65
				6213.33	15500	40.09	25	206.67	12.09	395	266.67	148.12
				286.67	1360	21.08				10575	14293.33	73.98
				576.67	1420	40.61				762.5	26666.67	2.86
				175.56	185	94.89				5687.5	32000	17.77
				151.11	3400	4.44				0	24320	0
				666.67	3150	21.16				7280	15466.67	47.07
				551.11	2650	20.79				2.5	26.67	9.38
				67.78	1180	5.74				560	380	147.37
				264.44	820	32.25				5360	35840	14.95
				124.44	230	54.11				0	17333.33	0
				722.22	1400	51.59						
				886.67	1580	56.12						
				212.22	260	81.62						
				296.67	1415	20.97						
				33.33	65	51.28						
				21.11	140	15.08						
				137.78	190	72.51						
				55.56	90	61.73						
				290	560	51.78						
				760	9493.33	8.01						
				4640	18560	25						
				10240	17280	59.26						
				3840	14933.33	25.71						



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5450	38826.67	14.04
367.5	1466.67	25.06
3760	3920	95.92
5200	5493.33	94.66
5140	5546.67	92.67
7560	9066.67	83.38

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**Table 2.1.2.** Total viable counts (TVC), counts of MRB in sediment (no. g<sup>-1</sup> dry wt.) from Goa during 1999-2001 and their percent in the TVC on seawater nutrient agar amended with 10 ppm mercury

Year	1999					2000*					2001*					
	no. of sediment samples	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
	22				5			10								
		2004.58	49828.18	4.02	0.22	33.48	0.67	383.27	12543.55	3.05	383.27	12543.55	3.05	383.27	12543.55	3.05
		0.63	4436.86	0.01	1527.27	556.82	274.23	501.49	8119.40	6.18	501.49	8119.40	6.18	501.49	8119.40	6.18
		2210.85	9822.65	22.51	1269.33	2360	53.78	50.59	319.57	15.83	50.59	319.57	15.83	50.59	319.57	15.83
		2436.04	11039.52	22.07	2987.43	3141.83	95.09	574.09	698.97	82.13	574.09	698.97	82.13	574.09	698.97	82.13
		1.21	109.29	1.11	0	61.24	0	378.25	265.96	142.22	378.25	265.96	142.22	378.25	265.96	142.22
		5.67	170.07	3.33				1303.05	368.05	354.04	1303.05	368.05	354.04	1303.05	368.05	354.04
		801.91	10294.12	7.79				719.61	1574.14	45.71	719.61	1574.14	45.71	719.61	1574.14	45.71
		85.47	649.35	13.16				786.01	474.60	165.61	786.01	474.60	165.61	786.01	474.60	165.61
		0	968.83	0				0	37.88	0	0	37.88	0	37.88	0	0
		72.12	298.25	24.18				4.08	8405.79	0.05	4.08	8405.79	0.05	4.08	8405.79	0.05
		1862.78	8441.56	22.07												
		0	89.37	0												
		1979.11	25384.62	7.79												
		3201.71	165421.6	1.93												
		4089.98	20368.1	20.08												
		23.62	78.7	30.01												
		1.22	330.8	0.37												
		531.27	2844.6	18.68												
		545.38	58598.73	0.93												
		984.61	99076.92	0.99												
		454.73	41646.09	1.09												
		1420.76	46630.24	3.05												

\* Counts of CFU in no. g<sup>-1</sup> dry wt. X 10,000, otherwise no. g<sup>-1</sup> dry wt. X 1000

**Table 2.2.1.** Total viable counts (TVC), counts of MRB in water (no. ml<sup>-1</sup>) from Mangalore, west coast, India during 1993-2000 and their percent in the TVC on seawater nutrient agar amended with <5ppm Hg prior to 1999 and with 10 ppm mercury from 1999 onwards

Year	1993				1997				1999				2000			
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	
no. of water sample	18		20				6					8				
	52	400	13	0.2	991.67	0.02	2320	11520	20.14	650	1733.33	37.5	1946.67	9.37		
	79.2	235	33.7	0.4	673.33	0.06	2550	16320	15.62	182.5	5333.33	36.37	1946.67	9.37		
	156.8	274	57.23	0.6	883.33	0.07	990	2160	45.83	1940	5333.33	36.37	1946.67	9.37		
	18.4	51	36.08	0.3	986.67	0.03	2700	3360	80.36	102.5	240	42.71	1946.67	9.37		
	52	492	10.57	0.2	700	0.03	5440	7253.33	75.00	175	306.67	57.06	1946.67	9.37		
	62.8	289	21.73	0.2	1416.67	0.01	2160	3840	56.25	27.5	166.67	16.5	1946.67	9.37		
				0.4	1116.67	0.04				430	9813.33	4.38	1946.67	9.37		
				0.6	1075	0.06				960	566.67	169.41	1946.67	9.37		
				0.2	575	0.03							1946.67	9.37		
				0.4	1393.33	0.03							1946.67	9.37		
				2	5500	0.04							1946.67	9.37		
				5	2068.75	0.24							1946.67	9.37		
				3	1177.5	0.25							1946.67	9.37		
				9	3450	0.26							1946.67	9.37		
				11	4125	0.27							1946.67	9.37		
				1	2600	0.04							1946.67	9.37		
				3.5	1875	0.19							1946.67	9.37		
				2	3750	0.05							1946.67	9.37		
				3	3750	0.08							1946.67	9.37		
				2.5	2812.5	0.09							1946.67	9.37		

**Table 2.2.2.** Total viable counts (TVC), counts of MRB in sediment (no. g<sup>-1</sup> dry wt.) from Mangalore during 1997-2000 And their percent in the TVC on seawater nutrient agar amended with <5ppm Hg prior to 1999 and with 10 ppm mercury from 1999 onwards

Year	1997			1999*			2000*			
	no. of samples in sediment	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
	20	0.68	155.93	0.44	804.9536	95975.23	0.84	2488.12	17278.62	14.4
		1.09	269.75	0.4	504.5872	107470.5	0.47	7690.25	23925.23	32.14
		1.12	47.592	2.35	800	71652.17	1.12	2685.01	31691.88	8.47
		4.60	368.24	1.25				45.63	5840.939	0.78
		4.769	152.59	3.12						
		3.482	214.74	1.62						
		2.341	74.16	3.16						
		4.971	55.90	8.89						
		2.146	214.59	1.00						
		0.732	152.59	0.48						
		0.153	49.79	0.31						
		0.54	184.00	0.29						
		2.91	352.00	0.83						
		2.00	163.00	1.23						
		0.19	128.00	0.15						
		4.43	403.00	1.09						
		11.83	784.12	1.51						
		2.35	151.25	1.55						
		3.02	191.67	1.57						
		2.93	410.56	0.71						

\* Counts of CFU in no. g<sup>-1</sup> dry wt. X 10,000, otherwise no. g<sup>-1</sup> dry wt. X 1000

**Table 2.3.** Total viable counts (TVC), counts of MRB in water (no. ml<sup>-1</sup>) and sediment (no. g<sup>-1</sup> dry wt.) from Gopalpur, East coast, India during 1999-2002 on seawater nutrient agar amended with 10 ppm mercury

Year	1999				2000				2002			
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
no. of water samples	16			8			6					
	81.2	110	73.82	56	150	37.33	717.5	2670	26.87			
	200	260	76.92	120	574	20.91	262.5	1375	19.09			
	30.4	34	89.41	76	536	14.18	1712.5	840	203.87			
	35.4	176	20.11	80	1152	6.94	470	585	80.34			
	660	1350	48.89	36	384	9.38	330	310	106.45			
	264	1032	25.58	92	578	15.92	990	1500	66			
	15	1064	1.41	64	8856	0.72						
	17.2	564	3.05	80	4352	1.84						
	80	110	72.73									
	160	260	61.54									
	5	34	14.71									
	1.6	176	0.91									
	500	1350	37.04									
	320	1032	31.01									
	3.4	1064	0.32									
	1.4	564	0.25									
Year	1999				2000				2002*			
no. of sediment samples	8			8			6					
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
	4031.7	7256.71	55.56	771.43	13200	5.84	102.90	58.80	175			
	7833.75	29797.8	26.29	914.29	6742.86	13.56	11.42	12.92	88.33			
	1930.58	55663.57	3.47	742.86	11857.14	6.27	3.41	36.74	9.29			
	910.58	941.27	96.74	874.29	25314.29	3.45	86.28	1084.07	7.96			
	2959.05	7256.71	40.78	542.86	13200	4.11	4.35	47.08	9.23			
	280.83	29797.8	0.94	800	6742.86	11.86	0.29	5.72	5			
	2016.27	55663.57	3.62	542.86	11857.14	4.58						
	895.23	941.27	95.11	800	25314.29	3.16						

\* Counts of CFU in no. g<sup>-1</sup> dry wt. X 10,000, otherwise no. g<sup>-1</sup> dry wt. X 1000

**Table 2.4.** Total viable counts (TVC), counts of MRB in water (no. ml<sup>-1</sup>) sediment (no. g<sup>-1</sup> dry wt.) from Chennai during 1996-2001 and their percent in the TVC on seawater nutrient agar amended with <5ppm Hg prior to 1999 and with 10 ppm mercury from 1999 onwards

Year	1996				1997				1999				2001			
	no. of water samples		9		10		5		13		13		13		2001*	
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	
	0	236.5	0	43.6	228.67	19.07	400	18113.33	2.21	2200	2208	99.64	2200	2208	99.64	
	0.5	89.5	0.56	55	378	14.55	200	25386.68	0.79	592.5	608	97.45	592.5	608	97.45	
	0	71.5	0	47.8	389.33	12.28	480	7433.33	6.46	27.5	512	5.37	27.5	512	5.37	
	0.5	250.5	0.19	48.5	320	15.16	300	15680	1.91	550	560	98.21	550	560	98.21	
	0.5	160	0.31	39	401.33	9.72	440	848	51.89	580	808	71.78	580	808	71.78	
	0	51.5	0	6	920	0.65				640	1200	53.33	640	1200	53.33	
	0.5	197	0.25	15	780	1.92				127.5	432	29.51	127.5	432	29.51	
	0.5	64	0.78	31	905	3.42				265	128	207.03	265	128	207.03	
	0	58	0	8	635	1.26				45	12	375	45	12	375	
				4.6	435	1.06				12.5	80	15.62	12.5	80	15.62	
Year	1996				1997				1999				2001*			
no. of sediment samples	9				9				3				11			
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB	
	0.002	12.46	0.016	3.79	375.92	1.01	320.28	2109.93	15.18	449.08	1231.76	36.46	449.08	1231.76	36.46	
	0	5.15	0	3.14	64.58	4.86	782.03	993.73	78.69	128.45	2279.92	5.63	128.45	2279.92	5.63	
	0	1.32	0	3.7	8.98	41.2	61.05	2246.00	2.72	194.63	476.43	40.85	194.63	476.43	40.85	
	0.001	13.41	0.007	1.33	76.04	1.75				130.25	1261.35	10.33	130.25	1261.35	10.33	
	0	6.08	0	4.83	43.07	11.21				233.13	1099.39	21.21	233.13	1099.39	21.21	
	0	5.61	0	1.3	70.4	1.85				228.69	797.39	28.68	228.69	797.39	28.68	
	0.003	9.56	0.031	3.2	513.1	0.62				522.43	1919.67	27.21	522.43	1919.67	27.21	
	0	3.27	0	3.2	25.3	12.65				96.86	1133.03	8.55	96.86	1133.03	8.55	
	0	3.31	0	0.8	99.97	0.8				147.59	512.79	28.78	147.59	512.79	28.78	
				0.8	99.97	0.8				213.29	793.17	26.89	213.29	793.17	26.89	

\* Counts of CFU in no. g<sup>-1</sup> dry wt. X 10,000, otherwise no. g<sup>-1</sup> dry wt. X 1000

**Table 2.5.** Total viable counts (TVC), counts of MRB in water (no. ml<sup>-1</sup>) and sediment (no. g<sup>-1</sup> dry wt.) from Ratnagiri during 1999-2001 and their percent to the TVC on seawater nutrient agar amended with 10 ppm mercury

Year	1999			2000			2001		
No. of water samples	6			10			16		
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
	2080	17280	12.04	7200	4026.67	178.81	1280	2666.67	48
	1420	13440	10.56	4800	833.33	576	14880	6533.33	227.75
	6430	13013.33	49.41	640	153.33	417.39	5320	873.33	609.16
	3440	9173.33	37.50	3120	1093.33	285.37	1210	1066.67	113.44
	8960	13013.33	68.85	590	506.67	116.45	2360	226.67	1041.18
	4320	6613.33	65.32	37.5	20	187.5	310	6.67	4650
				2100	2693.33	77.97	9600	7573.33	126.76
				19200	533.33	3600	1960	386.67	506.89
				1080	580	186.21	162.5	460	35.33
				760	693.33	109.61	187.5	680	27.57
Year	1999			2000*			2001*		
No. of sediment samples	3			5			8		
	MRB	TVC	% of MRB	MRB	TVC	% of MRB	MRB	TVC	% of MRB
	705.88	65882.35	1.07	533.49	5545.48	9.62	913.88	1028.12	88.89
	606.06	1165.5	52.00	971.43	5061.22	19.19	172.39	14557.06	1.18
	2046.78	21491.23	9.52	1373.96	1398.89	98.22	55.80	5783.13	0.96
				0	5714.29	0	0	8.11	0
				5.33	8187.63	0.06	497.07	2669.79	18.62
							0.50	336.25	0.15
							5.47	52.08	10.5
							11.33	1359.37	0.83

\*Counts of CFU in no. g<sup>-1</sup> dry wt. X 10,000, otherwise no. g<sup>-1</sup> dry wt. X 1000

**Table 2.6.** Total viable counts (TVC), counts of mercury resistant bacteria (MRB) capable of growth on seawater nutrient agar with 0.5 ppm Hg and, *in situ* concentrations of Hg from different locations along the Indian coast during 1993-1997

Location	Year (month)	Sample (n)	TVC range; (mean± SD)	MRB range; (mean± SD)	% of MRB in TVC	Hg conc. <sup>1</sup>
Chennai	1993 (Feb)	water (18)	40.5-220 <sup>2</sup> ; (122.05±75.94)	0-0.6; (0.23±0.3)	0.19	0.62-0.68
		sed (18)	0.6-12.45; (6.62±4.23)	0-0.03; (0.0003± 0.00015)	0.7	NA <sup>3</sup>
	1996 (May)	water (9)	51.5-250.5; (130±80.63)	0-0.5; (0.28±0.26)	0.23	42-145
		sed (9)	1.32-13.41; (6.68±4.22)	0-0.05; (0.0007±0.0001)	0.006	16-44
	1997 (Apr)	water (10)	228.67-920; (539±251.39)	6-55; (29.85±19.67)	7.9	42-145
		sed (9)	8.98-513.1; (141.93±177.05)	0.08-0.48; (0.28±0.14)	8.48	0-118
Mangalore	1993 (May)	water (18)	51-400; (290.16±150.54)	1.84-21.76; (11.37±5.86)	4.86	NA
		water (18)	47-848; (273.95±198.02)	0-10.53; (2.63±0.81)	0.96	10-112
	1996 (June)	sed (18)	0.002-2.08; (0.52±0.36)	0.0002-0.02; (0.005±0006)	0.96	NA
		water (20)	575-5500; (2046.02±1410.47)	0.2-11; (2.28±2.99)	0.09	17-177
	1997 (March)	sed (20)	47.59-784.12; 226.17±172.87	0.015-1.18; (0.28±0.26)	1.6	18-118
		water (6)	17-203; (79.75±64.25)	1.8-45.2; (6.62±5.08)	1.24	15-140
Mormugao	1996 (May)	water (12)	27-405.5; (92.24±78.06)	0-22.5; (7.12±3.8)	0.89	32-62
		sed (12)	40.46-2070.06; (508±492.6)	0.0003-0.005; (0.002±0.0007)	1.03	NA
	1993 (Feb)	water (6)	17-203; (79.75±64.25)	1.8-45.2; (6.62±5.08)	1.24	15-140
Positra	1996 (Sep)	water (14)	98-300.67; (165.55±54.85)	0-0.5; (0.09±0.14)	0.06	0-102.2
		sed (5)	2.77-8.37; (4.72±2.28)	0-0.01; (0.0004±0.0005)	0.14	22-101
	1997 (Feb)	water (14)	60-326; (160.87±71.82)	0-2.2; (0.26±0.58)	0.22	5-102
		sed (5)	14.97-41.49; (24.23±11.47)	0.009-0.03; (0.02±0.009)	0.65	22-71

<sup>1</sup>range of mercury concentration in water (ng l<sup>-1</sup>) and sediment (ng gm<sup>-1</sup> dry wt.) <sup>2</sup>TVC cells ml<sup>-1</sup> water and no x 10<sup>4</sup> g<sup>-1</sup> dried sediment (sed); <sup>3</sup>data not available



**Table 2.7.** Total viable counts (TVC), counts of mercury resistant bacteria (MRB) capable of growth on seawater nutrient agar with 10 ppm Hg and, *in situ* concentrations of Hg from different locations along the Indian coast during 1999-2002

Location	Year (month)	Sample (n)	TVC range; (mean±SD)	MRB range; (mean±SD)	% of MRB in TVC	Hg <sup>1</sup>
Paradip	2002 (Feb)	water (6)	310-2670 <sup>2</sup> ; (1213.33±846.21)	262.5-1712.5; (747.08±543.59)	83.8	NA <sup>3</sup>
		sed (6)	5.72-1084.07; (207.56±429.87)	0.28-102.9; (34.77±46.77)	49.13	NA
Gopalpur	1999 (Mar)	water (16)	34-1350; (573.45±492.07)	1.4-660; 148.41±197.75	34.86	2-117
		sed (8)	94.12-5566.36; (2341.48±2297.12)	28.08-783.37; (260.72-243.48)	40.30	72-128
	2000 (Mar)	water (8)	150-8856; (2072.75±3057.69)	36-120; (75.5±24.97)	13.40	NA <sup>3</sup>
		sed (8)	674.28-2531.43; (1427.86±728.21)	54.29-91.43; (74.86±13.82)	6.60	NA
Chennai	1999 (Nov)	water (5)	848-25386.68; (13492.27±9542.08)	200-480; (364±113.50)	12.65	100-2100
		sed (3)	9.93-224.6; (178.32±68.71)	6.1-78.20; (38.78±36.52)	32.20	237-338
Nagpattinam	2001 (Dec)	water (13)	12-2208; (590.77±610.28)	12.5-2200; (406.15±592.15)	95.99	0-788
		sed (11)	476.43-2279.92; (1150.49±577.58)	96.86-522.43; (234.44±141.28)	23.46	NA
Mangalore	1999 (Mar)	water (6)	2160-16320; (7408.88±5531.99)	990-5440; (2693.33±1475.80)	48.87	NA
		sed (3)	7165.21-10747.05; (9169.93±1828.80)	50.46-80.49; (70.32±17.20)	8.10	NA
	2000 (Mar)	water (8)	166.67-9813.33; (2513.33±3412.28)	27.5-1940; (558.44±640.41)	46.66	45-1088
		sed (8)	5840.94-31691.88; (19684.17±10948.27)	45.63-7690.25; (3227.25±3208.40)	13.95	133-172
Mormugao	1999 (Feb)	water (38)	65-38826.67; (4721.89±7592.84)	70-10240; (1815.26±2593.19)	44.82	152-456
		sed (22)	7.87-16542.16; (2529.67±4048.03)	0.001-408.99; (103.24±119.89)	9.32	53-194
	2000 (Mar)	water (10)	26.67-17386.67; (1957.33±2423.51)	25-5020; (704.45±1530.14)	92.39	NA
		sed (5)	33.48-3141.83; (1230.67±1430.28)	0-2987.43; (1156.85±1242.68)	84.76	NA
	2001 (Mar)	water (29)	6.67-35840; (11783±11058.93)	0-10575; (3588.28±3488.60)	73.48	NA
		sed (11)	37.88-20000; (4800.72±6662.64)	0-1303.05; (545.49±460.87)	74.67	NA
	2002 (Mar)	water (32)	2-1200; (247.87±336.64)	0-300; (97.95±103.67)	65.81	NA
		sed (2)	26611.23-27906.48; (27259.1±916.23)	8684.65-9069.77;	32.50	NA

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			(8859.21±217.78)			
Ratnagiri	1999 (Mar)	water (6)	6613.33-17280; (12088.89±3713.88)	1420-8960; (4441.67±2831.78)	40.61	22-103
		sed (6)	116.55-6588.23; (2951.30±3309.57)	60.6-204.68; (111.96±80.45)	20.86	44-208
	2000 (Mar)	water (10)	20-4026.67; (1113.33±1262.49)	12.5-2270; (747±663.06)	92.34	NA
		sed (5)	1398.89-8187.63; (5181.5±2438.02)	0-1373.96; (576.84±602.57)	25.42	NA
	2001 (Mar)	water (16)	6.67-7573.33; (1967.5±2361.32)	20-9350; (1553.91±2603.26)	92.40	NA
		sed (8)	8.11-14557.06; (3224.24±4961.79)	0-913.88; (207.06±332.15)	15.14	NA
Mumbai	2000 (May)	water (4)	16540-16660; (16040.5±16010)	16020-16057.5; (16040.5±16035)	97.00	180-440
		sed (3)	2375.84-2790.36; (2583.09±293.11)	97.31-800.58; (448.95±497.29)	16.39	210-1390
	2001 (Nov)	water (13)	270-6192; (1822.61±1517.24)	100-2410; (930.09±686.43)	58.14	NA
		sed (11)	2.65-2360; (468.52±663.12)	0-152.4; (22.4±43.93)	3.28	NA
	2002 (Apr)	water (3)	382.5-3785000; (1262224±2184788)	255-2380000; (793722.5±1373757)	66.76	NA
		sed (9)	12.33-482.04; (175.67±132.77)	9.02-452.37; (138.66±133.89)	70.80	NA

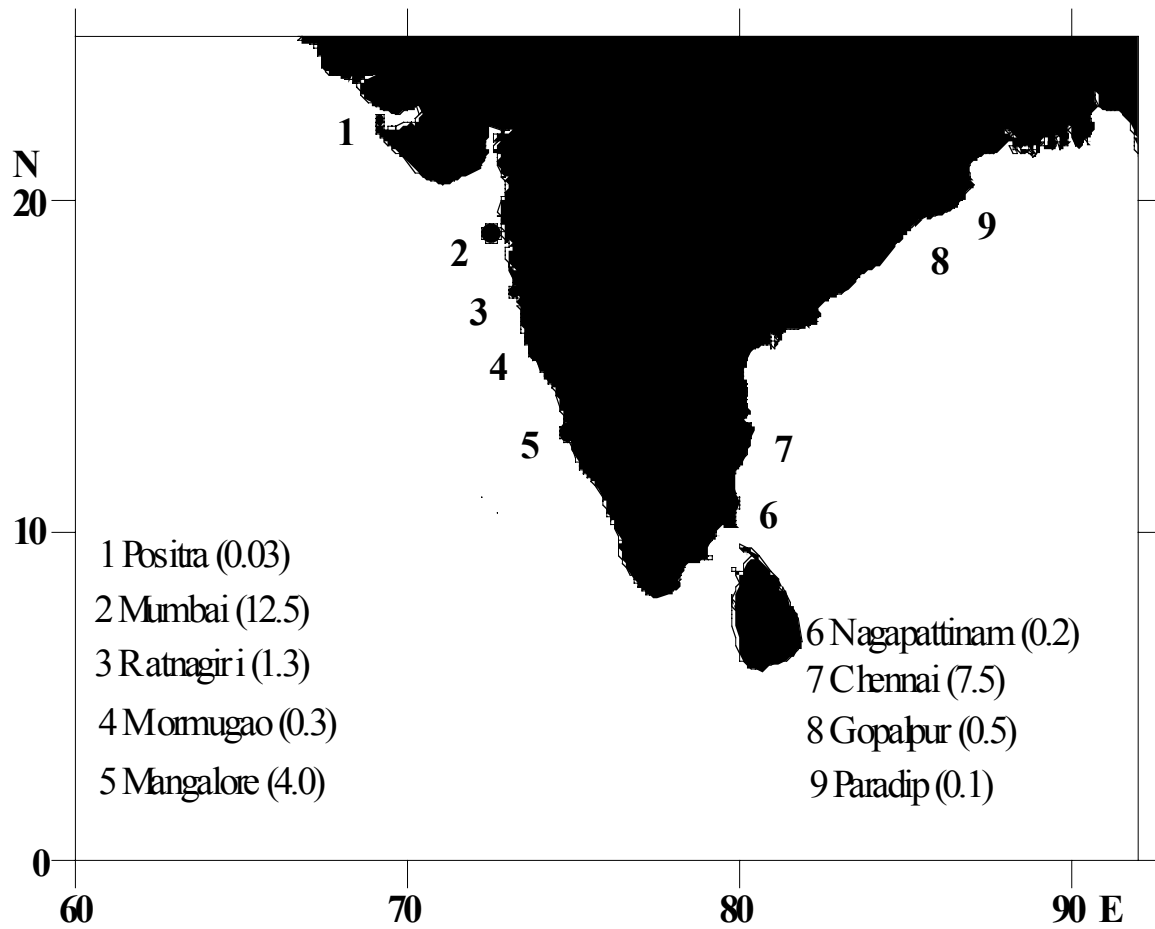
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<sup>1</sup>range of mercury concentration in water (ng l<sup>-1</sup>) and sediment (ng gm<sup>-1</sup> dry wt.), <sup>2</sup>TVC cells ml<sup>-1</sup> water and no x 10<sup>4</sup> g<sup>-1</sup> dried sediment (sed); <sup>3</sup>data not available

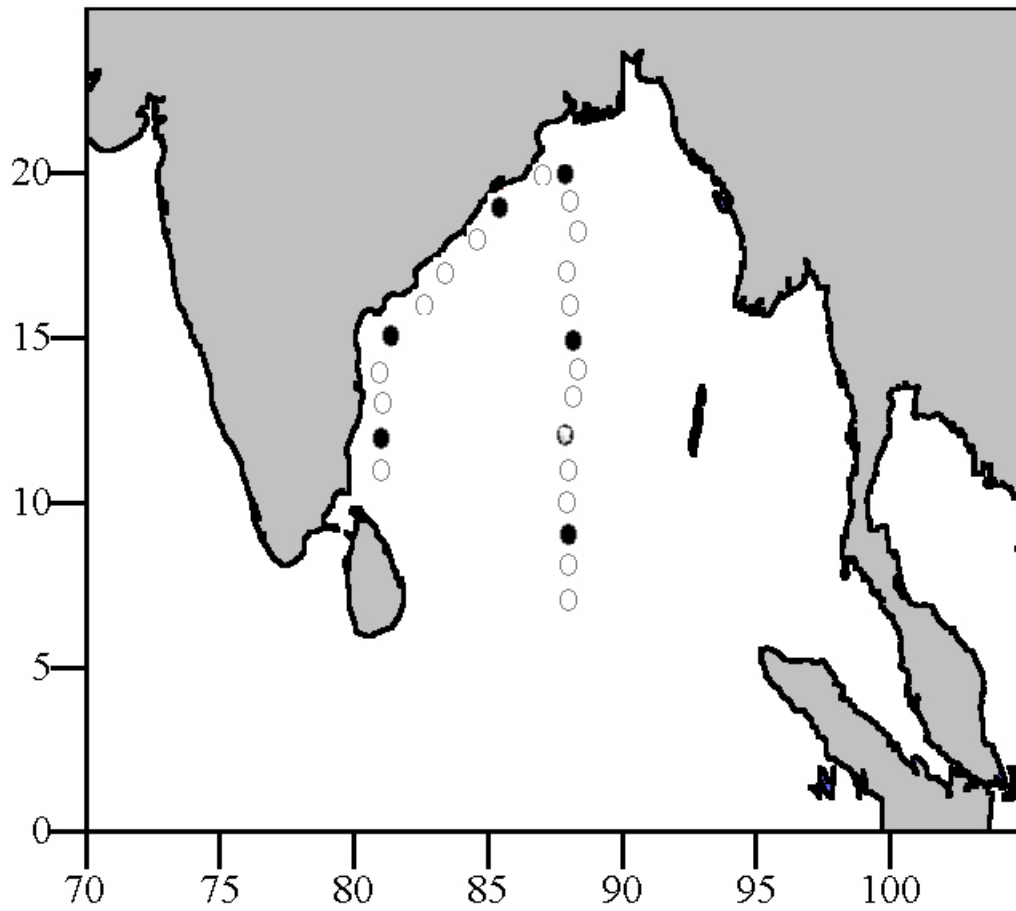
**Table 2.8.** Total viable counts (no. ml<sup>-1</sup>) from different locations from the Bay of Bengal sampled during July-August, 2001

Depth (m)	Oceanic stations			Coastal stations		
	9° N 88° E	15° N 88° E	20° N 88° E	19° N 85°E	15° N 81° E	12° N 82° E
1	260.00*	371.43	1265.71	154.29	8.57	22.86
10	204.29	94.29	205.71	52.86	7.14	20.00
20	115.71	434.29	2262.86	1414.29	21.43	108.57
40	55.71	210.00	3682.86	81.43	42.86	21.43
60	321.43	201.43	3245.71	64.29	10.00	14.29
80	10477.14	128.57	3417.14	1554.29	34.29	37.14
100	557.14	1091.43	118.57	380.00	5148.57	730.00
120	56.00	142.40	40.60	22.60	25.71	74.29
200	28.00	53.60	28.10	40.00	21.43	28.57
300	15.20	120.00	26.80	38.40	15.71	275.71
400	25.60	81.60	5.40	18.60	11.43	10.00
500	27.60	50.40	10.70	240.00	51.43	5.71
600	14.80	99.20	13.87	NS**	5.71	12.86
800	19.47	33.60	86.67	NS	1382.86	108.57
1000	41.60	NS	NS	NS	3017.14	662.86

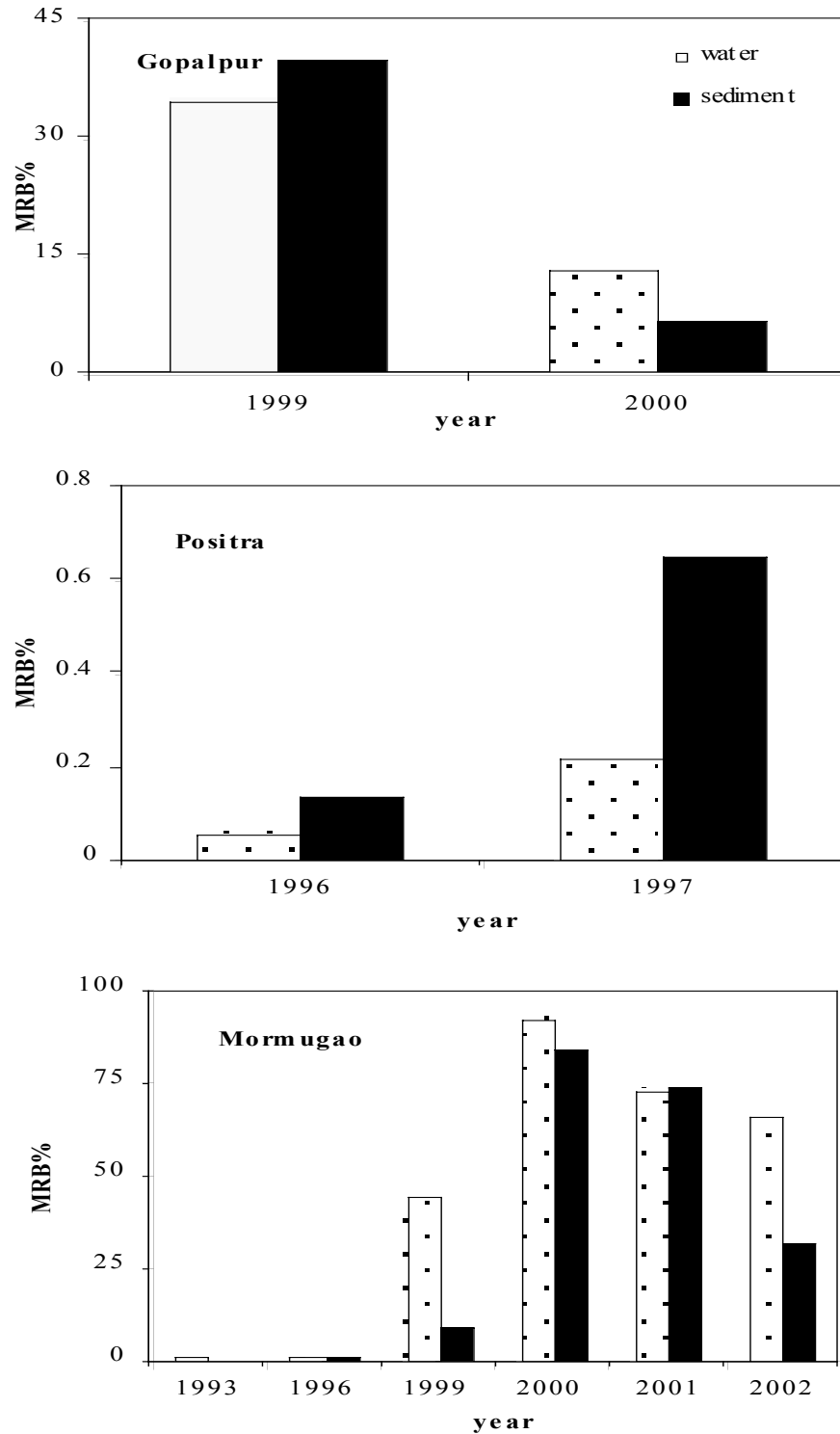
\*TVC (no. ml<sup>-1</sup>); \*\*NS, not sampled



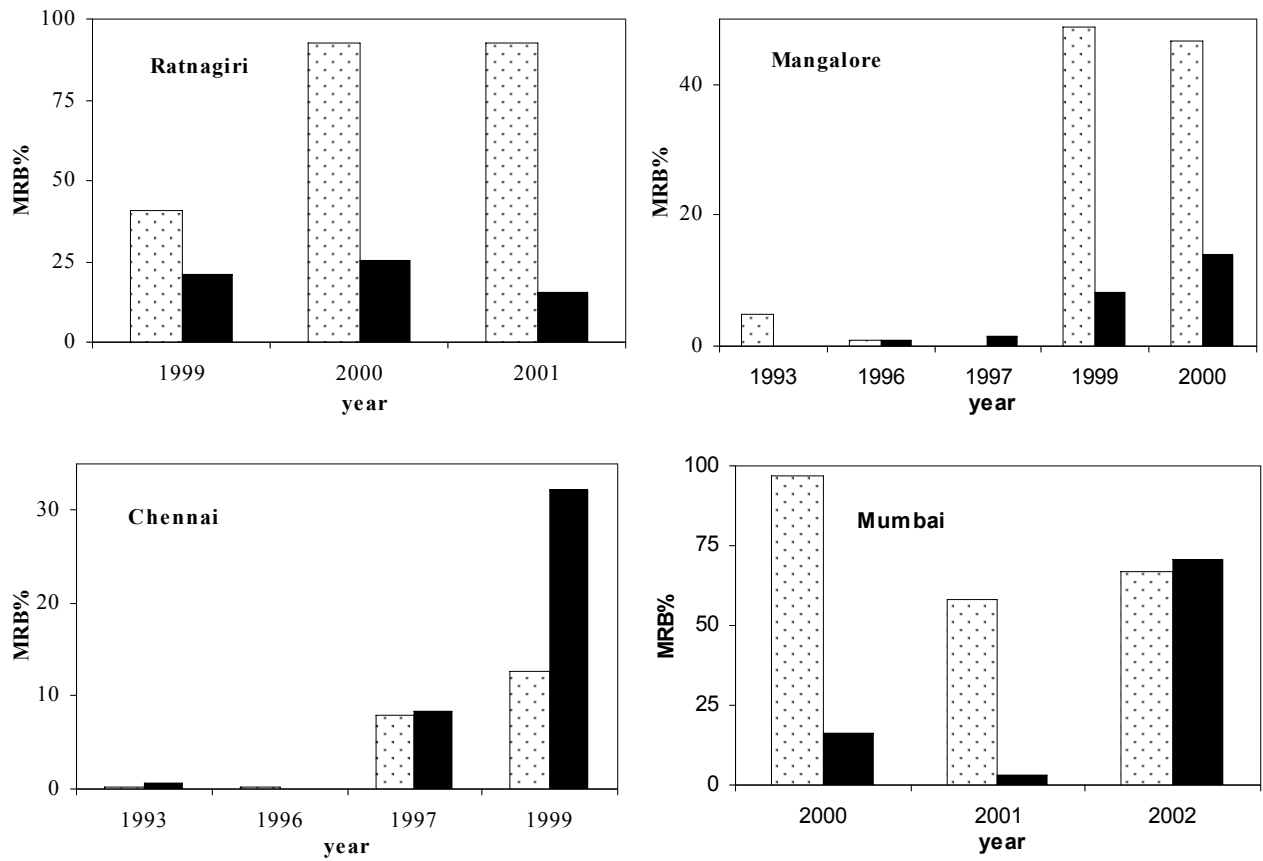
**Figure 2.1.** Location map of sampling stations along the Indian coasts. Approximate human population of the nearby coastal towns given in millions in parentheses



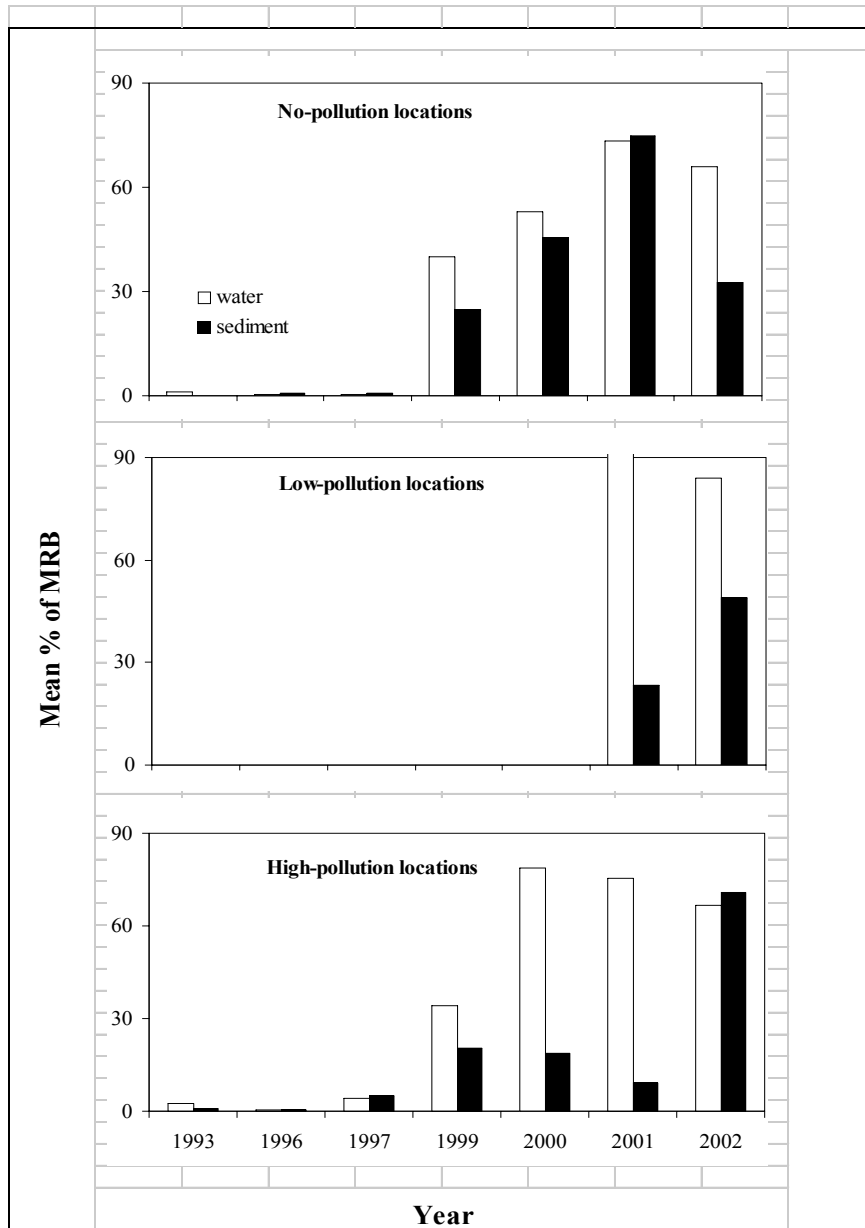
**Figure 2.2.** Cruise track of SK-166 during July-August, 2001. Sampling for enumerating mercury resistant bacterial populations carried out from stations marked with filled circle



**Figure 2.3.** Mean *percentage* of MRB (in total viable counts of bacteria) in water and sediment (solid bars) samples from no-pollution (Positra, Marmugao and Gopalpur) coastal locations to depict their *percent* increase during 1993-2002. See Tables 2.1 and 2.3 for complete data sets.

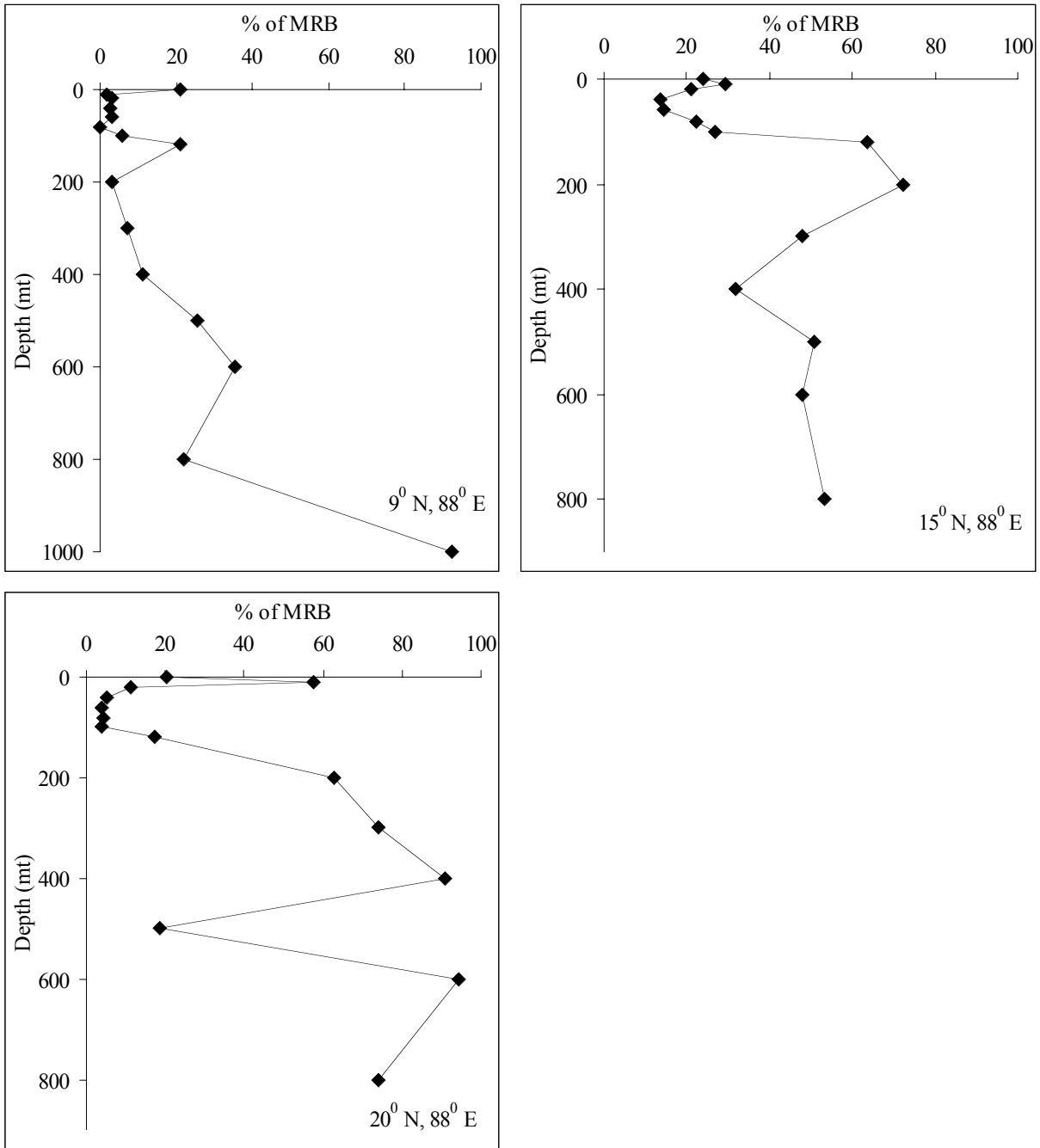


**Figure 2.4.** Mean *percentage* of MRB (in total viable counts of bacteria) in water and sediment (solid bars) samples from high pollution (Mumbai, Chennai, Mangalore and Ratnagiri) coastal locations to depict their *percent* increase during 1993-2002. See Tables 2.2, 2.4 and 2.5 for complete data sets.

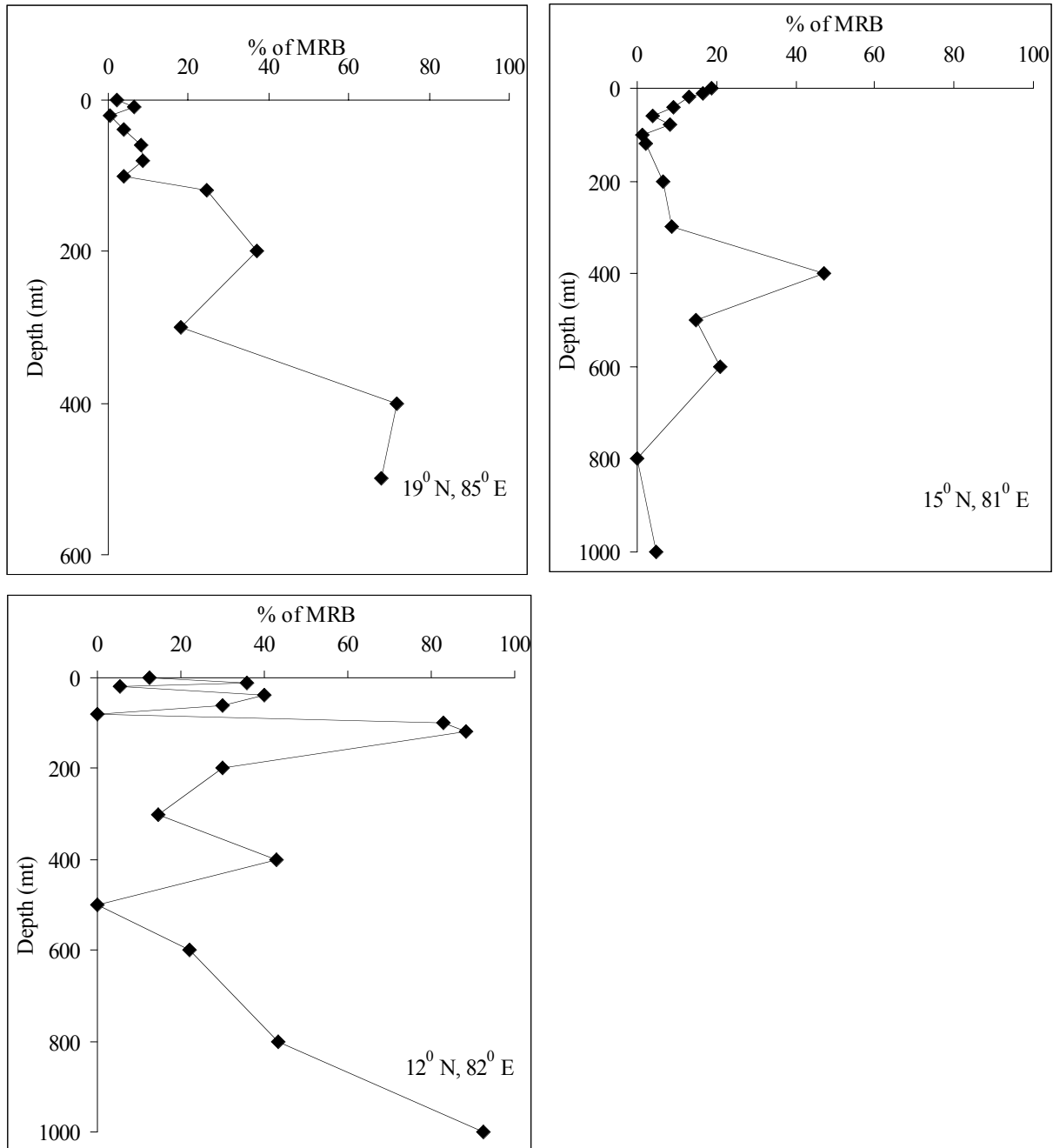


**Figure 2.5.** Mean percentage of MRB (in total viable counts of bacteria) in water and sediment (solid bars) samples. The MRB abundances pooled from no-pollution (Positra, Marmugao, Terekhol and Gopalpur) low pollution (Malvan, Karwar, Paradip, Nagpattinam) and high pollution (Mumbai, Chennai, Mangalore, Kulai, Padubidri and Ratnagiri) coastal locations to depict their *percent* increases during 1993-2002. See Tables 2.6 and 2.7 for complete data sets.





**Figure 2.6.** Vertical profiles of percent mercury-resistant bacteria in the oceanic stations in the Bay of Bengal. The total viable counts are presented in Table 2.8.



**Figure 2.7.** Vertical profiles of percent mercury-resistant bacteria in the coastal stations in the Bay of Bengal. The total viable counts are presented in Table 2.8

### **3.1. INTRODUCTION:**

Long-term exposure to heavy metals viz. Zn, Cu and Ni (Bååth et al., 1998) and Hg (Muller et al., 2001; Ramaiah & De, 2003) has been found to alter the microbial community. Certain environmental strains of bacteria have acquired highly specific resistance mechanisms for mercury. There is considerable evidence about mercury resistance among common microbial species (Canstein et al., 1999; Macalady, 2000; Pahan et al., 1990). Mercury resistant bacteria (MRB) are extremely important in detoxifying mercury compounds by two separate enzymes acting sequentially namely, organomercurial lyase that cleaves carbon-mercury bonds of certain organomercurials and mercuric reductase, which reduces  $\text{Hg}^{2+}$  to volatile mercury (Nakamura et al., 1990). Some bacteria have broad-spectrum resistance, i.e., to both  $\text{Hg}^{2+}$  and certain organomercurial compounds whereas some have narrow spectrum resistance and resistant only to  $\text{Hg}^{2+}$ . Under anaerobic conditions, bacteria are not usually harmed by mercury as it is changed directly into nontoxic mercury sulfide (Gerlach, 1981).

In order to biochemically characterize the MRB, a total of 83 isolates (60 from water and 23 from sediments) randomly isolated from different samples collected from coastal environments of India were chosen. They were maintained on SWNA amended with 10 ppm Hg as  $\text{HgCl}_2$  and taken up for detailed biochemical characterization. Thirty of these bacterial isolates were subjected to extensive morphological, physiological and nutritional characterization using different physiological and biochemical tests following MacFaddin (MacFaddin, 1980). A few studies (Barbieri et al., 1989, 1996; Ka et al, 1994) have examined marine MRB and their potential to catabolize toxic xenobiotics. In this chapter, characterization of mercury-resistant marine bacterial isolates, some of which grew well in the presence of 25 ppm ( $\geq 125 \mu\text{M}$ ) Hg (as

HgCl<sub>2</sub>) were designated as bacteria highly resistant to mercury (BHRM) were also examined for their biochemical characteristics. Thirteen of these isolates were subjected to 16S rDNA sequencing for their molecular identity.

## **3.2. MATERIALS AND METHODS:**

### **3.2.1. Isolation of MRB:**

Mercury resistant bacteria (MRB) in water and sediment complexes were isolated from various locations along the Indian coast during 1999-2002. Water (5 – 20 ml; membrane filtration technique using 0.22 µm pore sized membrane filter, Millipore, USA) and sediment (0.3 – 0.5 ml from 100X dilution; spread plating technique) samples were plated on seawater nutrient agar (SWNA: 5.0 g peptone, 1.5 g beef extract, 1.5 g yeast extract, 500 ml aged seawater, 500 ml deionized water and 15 g agar) amended with 10 ppm ( $\geq 50$  µM) mercury (as HgCl<sub>2</sub>). After 24-48 hours of incubation at the room temperature, enumeration of MRB was done and several single colonies were picked and streaked onto SWNA plates containing 10 ppm mercury for further purification. These isolates were found to have obligate requirement for sodium for their growth suggesting their marine origin (Baumann et al., 1972). All these MRB were checked for the resistance to a series of antibiotics. Although all of these isolates did not carry the typical *mer* genes, a number of them were shown to volatilize mercury (for details please see chapter 4 in this thesis). These purified isolates were further checked for growth on 25 ppm mercury. Based on colony characteristics, 30 isolates originating from coastal samples collected off Goa on the West Coast of India and off Chennai and Gopalpur on the East Coast were characterized and used in further experimentation.

### **3.2.2. Characterization of MRB:**

To characterize strains of MRB, several morphological, physiological and biochemical tests were carried out. All these tests were carried out following Cowan & Steel (1974) and MacFaddin (1980).

**Gram staining:** Freshly grown cultures were subjected to Gram staining and were observed under the microscope for their Gram reaction.

**Shape:** The shape of the bacteria was determined observing them under the microscope after subjecting to Gram staining.

**Motility:** Freshly grown liquid culture was examined under the microscope using grooved slide (hanging drop method) for checking motility of the bacteria.

**Enzyme profiles:** The isolates were grown on different substrates to check for lipase (Tween 80), gelatinase (gelatin), amylase (starch), oxidase, catalase (H<sub>2</sub>O<sub>2</sub>), and urease (urea) and decarboxylases (arginine/ornithine hydrochloride) activities by following standard microbiological methods described in MacFaddin (1980).

**Utilization of different carbon source:** The isolates were grown on modified complex liquid medium (Ramaiah, 1989) using different carbon sources (gluconate, pyruvate, citrate, cellobiose, glucose, sucrose, mannitol, arabinose, rhamnose) to check the ability of the MRB to use multitude of carbon source.

**Other tests:** The MRB isolates were also checked for nitrate reduction, methyl red (MR), Voges-Proskauer (VP) reaction, H<sub>2</sub>S production and oxidation-fermentation as described by MacFaddin (1980).

### **3.2.3. Identification of MRB using 16S rDNA sequencing:**

The isolates were grown on MB 2261 medium (Difco) supplemented with 1.5% agar (Difco). The genomic DNA extraction was done using the DNA extraction kit Nucleospin Extract (Macherey Nagel GmbH, Germany).

#### **3.2.3.1. Preparation of genomic DNA:**

##### ***Extraction of DNA:***

Approximately 50 mg (wet wt.) of each culture was scooped with sterile nichrome loop and transferred into 1.5 ml sterile eppendorf tube. One hundred and eighty (180)  $\mu$ l of T-1 (lysis buffer) and 8  $\mu$ l of RNAase were added to the tube using filter barrier tips, and the contents mixed thoroughly. The contents in the eppendorf tubes were mixed thoroughly by shaking for 30 minutes at 37° C on a thermomixer. Twenty five  $\mu$ l of Proteinase-K was added to each tube and the tube was then held at 56° C on a thermomixer for 30 minutes. An aliquot of 200  $\mu$ l of B-3 (Binding buffer) was added into each eppendorf tube and the eppendorf tube was heated for 10 minutes at 70° C. The tubes were further subjected to 95° C for 10 minutes for avoiding effect of excess RNAase. To this, 210  $\mu$ l of 98% ethanol was added and the tube was vortexed for 10-15 seconds. A Nucleospin column was placed on to a fresh 2 ml eppendorf tube and the mixture (now around 700  $\mu$ l) was transferred into the column, centrifuged for 1 minute at 9000 rpm. Five hundred  $\mu$ l buffer B-5 (wash buffer) was added to each column and centrifuged for 1 minute at 9000 rpm. The flowthrough was discarded. Again 500  $\mu$ l B-5 buffer was added to each column and centrifuged for an additional minute at 9000 rpm. The filtrate was discarded and the eppendorf tubes were centrifuged for 1 minute at 9000 rpm. One hundred  $\mu$ l preheated BE (elution buffer held at 70° C) was added to each column which were then held on the

thermomixer for 1 minute at 70° C. The eppendorf tubes were centrifuged at 9000 rpm for 1 minute, the columns were removed and the eppendorf tubes with eluted clean DNA were stored at -20° C.

### ***Gel electrophoresis:***

Purity of the extracted DNA was checked by electrophoresis through 0.8% TAE agarose gel and photographed using a gel-doc system (Herolab, Germany) following staining with ethidium bromide (1 µg/ml) for 10 minutes on a gel rocker platform and destaining in distilled water for 2 minutes. Gene ruler™ DNA ladder mix (MBI Fermentas) was used as marker and loading dye (6x loading dye solution, MBI Fermentas) was used to track the sample in the gel.

### **3.2.3.2. PCR amplification of 16S rDNA genes:**

The 16S rDNA gene was amplified from the sample DNA using oligonucleotide primers with specificity for eubacterial 16S rDNA genes (forward primer 8-27: 5'AGAGTTTGATCCTGG CTCAG 3' [modified from FD1] [Weisberg, 1991] and reverse primer 1492: 5'GGTTACCTTACGACTT 3' [Reysenbach, 1992]). The PCR was done in a thermocycler (PE, Applied Biosystems) using a sequencing program of 94 °C (1 min) for denaturing, 35 cycles of 94° C (0.10 min), 54° C (0.20 min), 68° C (1.30 min) with an extension temperature of 68° C (4.30 minutes). The amplified product was electrophoresed through 0.8% TAE-agarose gel, stained in ethidium bromide (1 µg/ml) and photographed using a transilluminator (Herolab, Germany). Gene ruler™ DNA ladder mix (MBI Fermentas) was used as marker and loading dye (6x loading dye solution, MBI Fermentas) was used to track the sample in the gel. The PCR product was cleaned using the QIAquick purification kit (Macherey Nagel GmbH, Germany).

***Cleaning of PCR product using QIAquick PCR-purification kit:***

Five volumes of buffer PB was added to the PCR product (e.g. 250  $\mu$ l of PB for 50  $\mu$ l PCR product). A QIAquick spin-column was placed into 2-ml eppendorf tube. The sample was transferred into this spin-column and spun for 1 minute at 14,000 rpm. The filtrate was discarded and 750  $\mu$ l of buffer PE was added to the column and spun for 1 minute at 14,000 rpm. The filtrate was discarded and the column spun again for 1 minute at 14,000 rpm. The column was placed into a 1.5 ml eppendorf tube; 30-50  $\mu$ l double distilled water was added into the column. After waiting for 1 minute, the eppendorf tube with the column was spun for 1 minute at 14,000 rpm for collecting the cleaned PCR product into the eppendorf tube which was later stored at -20°C until taken up for further analyses.

***Extension of PCR products:***

The cleaned PCR products were extended further using big dye sequencing protocol (big dye premix 4  $\mu$ l + big dye sequencing buffer 2  $\mu$ l + PCR product 5 $\mu$ l + forward/reverse primer 1  $\mu$ l + HPLC water 8  $\mu$ l=20  $\mu$ l) in a thermocycler using a program of 95 °C (0.20 min), 55°C (0.20 min) for 25 cycles and 60 °C (3.30 min). The extension product was purified further prior to sequencing.

***Purification of extension product (in single tube):***

Each sample (extension product) was made upto 100  $\mu$ l with HPLC water (e.g. add 80  $\mu$ l HPLC water to 20  $\mu$ l sample) and 10  $\mu$ l of 3M sodium acetate (NaAc), pH 4.6 was added to it and resuspended. Two hundred and fifty  $\mu$ l of 100% ethanol (at room temperature) was added to the sample and was vortexed for 15 seconds followed by a centrifugation at 15,000 rpm (at RT) for 15 minutes or at 14,000 rpm for 30 minutes and supernatant was removed and the pellet washed



with 250 µl 70% ethanol (RT). The eppendorf tubes were centrifuged again at 15,000 rpm (at RT) for 5 minutes or at 14, 000 rpm for 10 minutes. After removing the supernatant pellet was dried in a speedvac (for 3 minutes) and stored at -20°C. Thus all the samples were readied for sequencing. The sequencing was done using ABI sequencing instrument following the method of Sanger et al. (1977). The sequences were first edited using the software Chromas 1.45 and edited sequences submitted online, checked for their matches using FASTA and BLAST programs and the identification confirmed.

#### **3.2.4. Growth characteristics of MRB:**

The growth of seven MRB isolates in the presence and absence of mercury singly -and in combination with DDT and phenol- was examined in seawater nutrient broth (SWNB). Twenty-four hour old cultures of GP16, CH07, GP07, GP08, GP15, GP17 and GO01 were inoculated in replicates of three flasks into SWNB in three flasks with Hg concentrations of 0, 10 and 50 ppm. The flasks were incubated on a rotary shaker (180 RPM) at room temperature (28 °C) for 48 h. The growth was monitored by measuring the absorbance of broth culture at 660 nm every 6 h. The growth patterns of two isolates (CH07 and GP16) capable of growing either in 75 ppm Hg in SWNB, 100 ppm DDT in SWNB or 1000 ppm phenol in SWNB were determined in SWNB with 20 ppm Hg + 20 ppm Phenol + 10 ppm DDT in combination. In all cases, log transformed cell counts (number  $\Gamma^{-1}$ ) were plotted to draw growth curves.

#### **3.2.5. Resistance of MRB to toxic heavy metals and xenobiotics:**

The ability of MRB to grow in the presence of different toxic heavy metals (viz. Hg, Cd, Pb, Cu, Zn), pesticides (DDT, propiconazole, penconazole, pretilachlor, profenofos, metolachlor), and other xenobiotics (phenol, phenanthrene, benzene and trichloroethane) by adding these chemicals

at different concentrations to the growth medium (mostly SWNB) was checked. The pesticides were procured from Novartis, Goa and the stock was prepared in organic solvent like hexane and the inorganic metals salts and other chemicals were procured from Qualigens (Glaxo) Fine Chemicals and the stock solution was suitably prepared in sterilized distilled water or hexane. Seventeen MRB strains isolated from samples collected off Goa and Gopalpur were grown in SWNB containing xenobiotics either individually or in combination. Cultures were incubated at 28 °C for 2-14 days and inspected regularly for growth. The presence of growth was confirmed by streaking a loopful of liquid culture on to SWNA in cases where visible growth was not evident or, could not be discerned due to turbidity produced by some xenobiotics in SWNB. A reference strain, *Photobacterium leognathi* (ATCC 25521: PL3) unable to grow in medium containing  $\geq 1.0$  ppm Hg was included as a control to ascertain the Hg tolerance of environmental isolates studied.

### **3.2.6. Antibiotic sensitivity:**

As McArthur & Tuckfield (2000) observe, mercury pollution can contribute to increased antibiotic resistance. Further, as previously noted by Sant'ana et al. (1989), combined resistance to both antibiotics and mercury in environmental isolates may be due to selection pressure as a consequence of existence of these toxicants in an environment. Thus, an array of antibiotics such as kanamycin, streptomycin, neomycin, tetracycline, ampicillin, chloramphenicol, Nalidixic acid, gentamycin and penicillin G by six isolates (CH03, CH07, CH12, GP06, GP15 and GP16) was tested to evaluate the antibiogram of these MRB, which were used in other detailed experiments. Antibiotic disc sensitivity assay was carried out as follows. 24-h old broth culture was spread on SWNA plates (as lawn culture) and antibiotic discs were placed on the fresh lawn cultures and the plates were incubated at room temperature for 24 hours. Diameters of the clear zones around

the discs were measured and the sensitivity of the isolates to particular antibiotic determined according to the chart provided by the manufacturer (Himedia, Mumbai).

### **3.2.7. Effect of Hg on Carbon:nitrogen (C:N) ratio:**

Effect of Hg on carbon: nitrogen (C:N) ratio was examined to realize whether such basic physiological alterations are brought about by this toxic heavy metal. To check if C:N ratios were altered during Hg detoxification by MRB, the C:N ratios were monitored for three strains of MRB grown in SWNA containing 0, 10 and 50 ppm Hg. The cells were harvested and dried at 65 °C to completely remove the moisture, as noted by attaining constant dry weight, usually by about 7 days, and to attain stabilized dry wt. The C:N ratios from triplicate samples for each strain were determined in a carbon, nitrogen and sulfur analyzer that uses a combustion step and thermo-conductivity detection of electric signal proportional to carbon, nitrogen and sulfur. The standard protocol supplied by the manufacturers was followed for determining the C:N ratios (NCS 2500, CE Instruments, ThermoQuest Italia, S. P. A., Milan).

## **3.3. RESULTS:**

### **3.3.1. Bacterial isolates:**

Examination of Hg resistance in natural isolates: A total of 120 isolates (85 from water and 35 from sediment) randomly picked up from SWNA without any added Hg were checked for mercury resistance by streaking them on to SWNB with 10 ppm Hg. Out of these, 83 of them (60 from water and 23 from sediment) were found to be mercury-resistant bacteria (i.e. capable of growing in presence of 10 ppm Hg<sup>2+</sup>). When all these 83 were streaked on to SWNB with higher concentrations of Hg, 30 could grow well on SWNB with 25 ppm, 21 in SWNB with 50 ppm Hg

(Table 3.1). For further studies, 30 isolates were chosen for identification and all other experiments during this study. In all, 17 isolates were from Gopalpur and Goa region and 13 isolates from Chennai. Based on the biochemical characteristics, Bergey's manual (1984) and Oliver's (1982) scheme there were 13 *Pseudomonas* (GO02, GO03, GO04, GO05, GP11, GP12, GP14, GP16, CH04, CH05, CH07, CH11, CM45), 5 isolates each of *Proteus* (GP06, GP07, GP09, GP10, GP13), and *Xanthomonas* (GP08, CH13, S3, MSM4, MSM8), four isolates of Enterobacteriaceae (GP17, CH01, CH03, CH12) and, one each of *Aeromonas* (GP15), *Alteromonas* (CM10) and unidentified (GO01) isolates. From these 30 isolates, 13 were picked up for 16S rDNA analyses for molecular characterization.

### **3.3.2. Characteristics of MRB:**

The isolates showed varied response to different biochemical tests and also in their morphological characters. Twenty six of the 30 isolates were gram negative only four were gram positive (Tables 3.2.1 & 3.2.2). The isolates were short or large rods in shape. GO01 was the only non-motile amongst the 30 isolates. The isolates showed presence of various enzymes but there was a distinct difference in between the isolates of the same group. Most (70%) of the Pseudomonads, 28% of the *Alcaligenes* and all of the *Proteus* sp. were oxidase positive. All the isolates identified as *Proteus* sp. were positive for lipase, gelatinase, amylase (Tables 3.2.3 & 3.2.4). Most of the isolates were catalase positive but lacked decarboxylases. Twenty of the isolates possessed urease activity and, most were able to utilize a wide range of substrate as carbon source (Tables 3.2.3 & 3.2.4). Nearly 50% (14 out of 30) of the isolates were, interestingly enough, unable to utilize glucose as sole source of carbon. Out of the 16 isolates that were able to utilize glucose, 10 were fermentative and six, oxidative (Tables 3.2.3 & 3.2.4).

### **3.3.3. 16S rDNA sequence analyses:**

Biochemical and 16S rDNA sequences analyses-based identification of the thirteen isolates is given Table 3.3 and the result is documented on gel in plate 3.1. Except in the case of CH07, results from 16S rDNA sequence analysis revealed a nearly completely different identity from that deduced by biochemical methods. Of the twelve isolates, seven were found to be *Alcaligenes faecalis* (GO01, GO02, GP06, GP14, GP15, GP16 and GP17), three *B. pumilus* (GP08, CH13 and S3) one *Bacillus* sp. (CM10) and one *Brevibacterium iodinium* (GP13).

### **3.3.4. Growth characteristics of MRB:**

Growth curves of seven isolates of BHRM, including one representative of all the genera identified from the biochemical study, suggest that there is a prolonged lag phase in the presence of Hg (Figure 3.1). Once cells enter the log phase, in some cases a steep increase follows a long time lapse showing diauxic growth (e.g. GP16). Generation times were far longer in the presence of 50 ppm Hg than 10 ppm Hg at least in the initial 48 h. For example, in the case of isolate GP16, which was identified as *Alcaligenes faecalis* through 16S rDNA analysis, there was only one doubling during the first 48 h whereas it attained stationary phase within 48 h in the medium without added Hg. Growth of CH07 (*Pseudomonas aeruginosa*) was much faster i.e., two generations within the first 6 h at 50 ppm Hg concentration. After a minor lag, its generation was rapid and attained nearly 161 generations between 54 and 72 h before entering stationary phase around 108 h. GP15 (*A. faecalis*) grew the slowest followed by GP07 in the SWNB with 50 ppm Hg. The growth patterns of the two isolates tested in the presence of Hg, DDT and phenol were also diauxic. When compared their growth in SWNB with 50 ppm Hg, the growth rate of both the strains was lower in the SWNB containing all three toxicants viz. Hg, phenol and DDT (Figure 3.1).

### **3.3.5. Resistance to toxic heavy metals and xenobiotics:**

For all experiments during this study, the MRB isolates capable of tolerating at least 25 ppm Hg were used and were termed bacteria highly resistant to mercury (BHRM). The ability of these BHRM isolates to grow in the presence of various toxic chemicals was highly variable (Table 3.4.1 & 3.4.2). For example, not all isolates identified as *Pseudomonas* or *Alcaligenes* highly resistant to mercury were able to grow in SWNB containing 50 ppm Hg (Table 3.4.1) or 1000 ppm phenol or 10% (v/v) trichloroethane (TCE). Similarly, there were differences in the growth responses of BHRM identified as *Proteus* and *Alcaligenes* (in case of penconazole). A few isolates were able to grow on phenanthrene or benzene as sole carbon sources (Table 3.4.2). GO01 (*A. faecalis*) was the only isolate, which was unable to grow at 50 ppm phenol (Table 3.4.2). When various toxicants were added together, growth of almost all isolates was very poor or absent (Table 3.4.3) suggesting that the tolerance level of BHRM is affected when many xenobiotics are present simultaneously. None of the BHRM strains tested during this study was able to tolerate and grow in the presence of formaldehyde (final concentration, 2% v/v) or formic acid (2% v/v).

### **3.3.6. Antibiotic sensitivity:**

All six strains (tested along with a known sensitive strain *E.coli*) examined for antibiotic sensitivity were resistant to most of the antibiotics tested. Except for GP15 all other isolates were sensitive to gentamicin. CH07 was sensitive also to kanamycin and chloramphenicol whereas GP06 was sensitive to neomycin besides being sensitive to gentamicin as evidenced from the inhibition zone (Table 3.5).

### **3.3.7. C:N ratio:**

The C:N ratios were observed to vary with different concentrations of Hg in the three strains tested (Table 3.6). An increase in these ratios with increasing Hg concentration was discernible in the case of two of the three tested isolates of BHRM. Whereas, in the case of CH07 (*P. aeruginosa*), there was a decrease in C:N. One-way analysis of variance (ANOVA), done to check if there were significant differences in the C:N ratios with varying Hg concentrations revealed statistically significant changes between controls and treatments with Hg (Table 3.6).

### **3.4. DISCUSSION:**

Several researchers have reported about detoxification of mercury by resistant bacteria (Wood et al., 1968; Summers & Silver, 1978; Nakamura & Nakahara, 1988; Nakamura et al., 1990; Brown et al., 1991; Osborn et al., 1997). Many bacterial species have been shown to develop resistance to mercury and other heavy metals (Silver & Phung, 1996; Meinhardt et al., 1997; Osborn et al., 1997; Ravel et al., 1998a, 1998b, 2000a, 2000b; Nies, 1999). Such organisms become important in continuing the basic biological processes in contaminated habitats. The reported genera to possess resistance to mercury mostly at 10 ppm or lower levels are, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Bacteriodes*, *Beijerinckia*, *Chromobacterium*, *Citrobacter*, *Clostridium*, *Cephalosporium*, *Deinococcus*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Erwinia*, *Flavobacterium*, *Klebsiella*, *Micrococcus*, *Moraxella*, *Morganella*, *Mycobacterium*, *Paracoccus*, *Planococcus*, *Proteus*, *Rhodococcus*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Xanthomonas*, *Hyphomonas*, *Thiobacillus*, *Vibrio* and *Yersinia* (Baldi et al., 1989; Robinson and Tuovinen, 1984; Osborn et al., 1997; Nascimento, 2003). During this study six different genera viz. *Pseudomonas*, *Proteus*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Xanthomonas* and few strains

of Enterobacteriaceae were identified. Although the pathways of Hg modifications have not yet been completely understood, the isolates of BHRM investigated during this study have many unique characteristics as detailed below.

For instance, there were deviations within the MRB strains assignable to the same genus on the basis of weighted percent (i.e.,  $\geq 80\%$ ) similarity in their biochemical characteristics and physiological responses. Capacity to utilize a wide variety of carbon sources suggests these bacteria possess nutritional versatility and can, with their abilities to deal with toxic substances, proliferate even when environmental conditions are not ideal/favourable. Requirement of NaCl (Baumann et al., 1972) for growth by marine bacteria has been evident from these isolates and this might probably throw some light on the mercury resistance mechanism of these MRB. As reported by Selifonova & Barkay (1994),  $\text{Na}^+$  in the marine environment might play a role in the transport of the ionic mercury across the cell membrane and thereby facilitate the reduction/other mechanism of detoxification.

The combined toxicity from different toxicants may adversely affect the growth rates of BHRM as was evidenced during this study. Environmental isolates that grew in the presence of 50 ppm Hg, due probably to genetically conferred resistance, were also able to grow at very high concentrations of other heavy metals like Cd, Pb as well as xenobiotics including TCE (considered as an environmental hormone), DDT, other pesticides, PCBs and phenol. However, in the combined presence of Hg, phenol and DDT, only a few isolates were able to grow. This suggests that most strains are unable to tolerate the additive toxicity of many toxicants in combination beyond certain limits but these results support the need for exploring the microorganisms for their role in mitigating environmental pollution problem.



Mercury pollution can contribute to increased antibiotic resistance (McArthur & Tuckfield, 2000). Most strains examined in this study were resistant to chloramphenicol and as Allen et al. (1977) proposed, resistance to chloramphenicol might be linked with heavy-metal resistance. Many workers have claimed that Hg-reductase activity in Hg-resistant bacterial cells is always inducible and never constitutive (Summers, 1986). Such findings from this study corroborate well with Rasmussen & Sørensen, (1998) that in mercury-polluted environment, frequencies not only of mercury resistance but also of antibiotic resistance are elevated among indigenous bacteria.

The significantly increased C:N (low N levels per unit cell mass) ratios in two of the three strains suggest the possibility of certain MRB species/strains producing copious amounts of extracellular enzymes or polysaccharides (EPS) to protect the cell (s) from toxic substances. The negatively charged EPS can bind mercury by adsorbing the inorganic mercury (Dong et al., 2000) and the enzymes act upon the Hg ions. Or, on the other hand, as in the case of one pseudomonad strain, CH07, where the changes in C:N ratios showed a decrease suggesting that the Hg ions are transported inside the cell and reduced by the action of *merA*. Transforming these ions to gaseous form, which, as Summers (1986) reported, will escape out of the cells due to high vapour pressure. Further, from the lower numbers of generations per unit time and the extended lag phases in all isolates tested in this study, it is possible to suggest that the formation of extracellular polysaccharides and enzyme-mercury complexes reduce growth rates in these strains. From the decreasing C:N ratios and faster growth rates of CH07 (*Pseudomonas aeruginosa*), it is discernible that Hg ions are allowed an entry inside the cell and may undergo intracellular physiological sequestration. From the significant changes in these ratios in all three

strains we examined, it is suggested that MRB undergo various physiological alterations to deal with Hg and other toxic substances. Moreover, this study is quite important to suggest that underlying mechanisms in MRB are various and they can function in well coordinated fashion rendering the resistant bacteria capable of detoxifying high concentration of mercury which is otherwise toxic for the cell.

The strains studied during this investigation exhibited the greatest resistance to mercury than any gram-negative marine environmental isolates reported so far. Further, these strains are far more resistant to mercury than the wild type *Deinococcus radiodurans* (Brim et al., 2000). Such environmental strains are of practical interest to microbiologists not only to revalidate present concepts of Hg resistance by native microflora but also to understand the evolution and significance of Hg resistance. Moreover, these strains even when grouped together, showed various differences in their biochemical characteristics, which warrant a close look at their genetic make up for a clearer understanding of mercury-resistance in marine bacteria.

**Table 3.1.** Number of bacterial isolates picked up from seawater nutrient agar (SWNA) without Hg and checked for growth on SWNA with different concentrations of Hg

no. of isolates (120)	number positive for growth		Identity of the isolates
from SWNA without Hg*	in 10 ppm Hg (83)	in 25 ppm Hg (30)	<i>Pseudomonas</i> , 13** <i>Proteus</i> , 5
85 from water	water (60)	water (21)	<i>Xanthomonas</i> , 5
35 from sediment	sediment (23)	sediment (9)	Enterbacteriaceae, 4 <i>Alteromonas</i> , 1 <i>Aeromonas</i> , 1 Unidentified, 1

\*Bacterial isolates were from water and sediment samples collected off Marmugao, Chennai and Gopalpur; \*\*number of isolate belonging to different genera

**Table 3.2.1.** Gram reaction and morphological characters of MRB (from Goa and Gopalpur)

Identity	<i>Proteus</i>																
	* UN																
Code	GO02	GO03	GO04	GO05	GP11	GP12	GP14	GP16	GP06	GP07	GP09	GP10	GP13	GP15	GP17	GP08	GO01
16S identity**	AF	ND	ND	ND	ND	ND	AF	AF	AF	ND	ND	ND	BI	AF	AF	BP	AF
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Shape	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR	LR	SR
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

\*Identity of isolates as derived from biochemical tests; \*\* Identity of isolates on the basis of 16S rDNA sequence and gene library comparisons; SR, small rod; LR, large rod; ND, not done; AF, *Alcaligenes faecalis*; BI, *Brevibacterium iodinium*; BP, *Bacillus pumilus*; EN, Enterobacteriaceae; XM, *Xanthomonas*; AM, *Aeromonas*; UN, unidentified; +, growth; -, no growth

**Table 3.2.2.** Gram reaction and morphological characters of MRB (from Chennai & other areas)

Identity*	<i>Pseudomonas</i>										AT		
	EN					XM							
Code	CH04	CH05	CH07	CH11	CH11	CH01	CH03	CH12	CH13	S3	MSM4	MSM8	CM10
16S identity**	ND	ND	PA	ND	ND	ND	ND	ND	BP	BP	ND	ND	B.sp.
Gram stain	-	-	-	-	-	-	-	-	+	+	-	-	+
Shape	SR	SR	SR	SR	SR	SR	SR	SR	SR	LR	SR	RD	LR
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+

\*Identity of isolates as derived from biochemical tests; \*\* Identity of isolates as derived from 16S rDNA sequencing; SR, small rod; LR, large rod; ND, not done; PA, *Pseudomonas aeruginosa*; BP, *Bacillus pumilus*; B.sp, *Bacillus* sp., EN, Enterobacteriaceae; XM, *Xanthomonas*; AT, *Alteromonas*; +, growth; -, no growth

**Table 3.2.3.** Biochemical characters of MRB (from Goa & Gopalpur)

Identity*	<i>Proteus</i>																				
	<i>Pseudomonas</i>		AM																	EN	XM
Code	GO02	GO03	GO04	GO05	GP11	GP12	GP14	GP16	GP06	GP07	GP09	GP10	GP13	GP15	GP17	GP08	GO01				
Gelatinase	-	+	-	-	+	+	-	-	+	+	+	+	+	-	-	-	+				
Lipase	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-				
Amylase	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-				
Arginine	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
Ornithin	-	-	-	+	-	-	-	-	+	+	+	+	+	-	+	-	-				
Oxidase	+	-	-	+	+	-	-	-	+	+	+	+	+	-	-	+	-				
NO <sub>3</sub> <sup>-2</sup> red <sup>n</sup>	-	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+				
Catalase	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-				
Urease	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-				
VP	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
MR	-	+	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-				
H <sub>2</sub> S	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-				
Indole	+	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+				
OF	NR	NR	O	NR	NR	O	O	O	F	F	F	F	F	O	F	F	NR				
Utilization of																					
Citrate	+	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-				
Gluconate	+	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+	-				
Pyruvate	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	+	-				
Cellobiose	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-				
Sucrose	AL	NR	A	A	NR	A	A	A	A	A	A	A	A	A	A	A	NR				
Mannitol	AL	A	A	AL	NR	AL	AL	AL	A	A	A	A	A	AL	A	A	AL				
Rhamnose	AL	NR	A	AL	AL	AL	AL	AL	AL	AL	AL	AL	AL	AL	AL	A	AL				
Arabinose	A	NR	AL	AG	A	AL	AL	AL	AL	AL	AL	AL	AL	AL	AL	A	AL				

\*Identity of isolates as derived from biochemical tests. A, acidic reaction; AL, alkaline reaction; O, oxidative; F, fermentative; VP, Voges Proscauer; MR, Methyl red; NR, no reaction; NT, not tested; ND, not done; EN, Enterobacteriaceae; XM, *Xanthomonas*; AT, *Alteromonas*; AM, *Aeromonas*; +, growth; -, no growth

**Table 3.2.4** Biochemical characteristics of MRB (from Chennai & other areas)

Identity*	<i>Pseudomonas</i>										EN		XM			AT	
	CH04	CH05	CH07	CH11	CM45	CH01	CH03	CH12	CH13	S3	MSM4	MSM8	CM10				
Gelatinase	-	+	+	+	+	-	-	-	-	+	+	-	+				
Lipase	+	-	-	-	-	-	+	+	+	-	-	+	-				
Starch	-	-	+	-	+	-	-	+	-	-	-	-	+				
Arginine	-	+	+	-	-	+	+	+	-	-	+	-	-				
Ornithin	-	-	-	-	-	+	+	+	-	-	-	-	-				
Oxidase	+	-	-	-	-	-	-	-	-	-	-	-	+				
Nitrate	+	+	+	+	+	+	+	+	-	+	-	-	-				
Catalase	+	++	++	++	+	+	+	+	++	+	+	++	+++				
Urease	-	+	+	-	+	+	+	+	-	+	-	-	+				
VP	-	-	-	-	-	+	+	-	-	-	-	-	-				
MR	-	-	-	-	-	-	-	+++	-	+	+	-	-				
H <sub>2</sub> S	-	-	-	-	-	+	-	-	+	-	-	-	-				
Indole	-	-	-	-	-	-	+	+	+	-	-	+	-				
OF	NR	NR	NR	O	NR	F	F	F	NR	NR	O	NR	NR				
Utilization of																	
Citrate	-	+	+	++	-	+++	+++	+	+	-	+++	-	-				
Sucrose	A	AL	AL	A	A	A+	A+	A+	NR	A	AL	AL	NR				
Mannitol	NR	A+	A	AL	NR	A	A	A+	NR	A	AL+	AL	A+				
Rhamnose	AL+	AL+	AL+	AL	NR	A+	A+	AL	NR	A	AL	AL	A				
<b>Arabinose</b>	AL+	A	A	A	NR	A±	A+	A+	AL	AL+	A	AL	NR				

\*Identity of isolates as derived from biochemical tests. A, acidic reaction; AL, alkaline reaction; O, oxidative; F, fermentative; VP, Voges Proscauer; MR, Methyl red; NR, no reaction; NT, not tested; ND, not done; EN, Enterobacteriaceae; XM, Xanthomonas; AT, *Alteromonas*; +, growth; -, no growth

**Table 3.3.** 16 S rDNA identity of MRB (compared with FASTA):

Code	Sequence match (%) - forward strand	Sequence match (%) - reverse strand	Identity on the basis of 16S rDNA	Identity on the basis of biochemical characteristics
GO02	99.59	100	<i>Alcaligenes faecalis</i>	<i>Pseudomonas</i> sp.
GP06	99.58	99.78	<i>Alcaligenes faecalis</i>	<i>Proteus</i>
GP08	100	99.74	<i>Bacillus pumilus</i>	<i>Xanthomonas</i>
GP13	98.54	99.02	<i>Brevibacterium iodinium</i>	<i>Proteus</i>
GP14	98.94	100	<i>Alcaligenes faecalis</i>	<i>Pseudomonas</i>
GP15	99.56	99.79	<i>Alcaligenes faecalis</i>	<i>Aeromonas</i>
GP16	99.65	100	<i>Alcaligenes faecalis</i>	<i>Pseudomonas</i>
GP17	99.56	100	<i>Alcaligenes faecalis</i>	Enterobacteriaceae
CH07	100	100	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> sp.
CH13	100	99.75	<i>Bacillus pumilus</i>	<i>Xanthomonas</i>
CM10	96.08	99.48	<i>Bacillus</i> sp.	<i>Alteromonas</i>
S3	99.77	99.77	<i>Bacillus pumilus</i>	<i>Xanthomonas</i>
GO01	100	100	<i>Alcaligenes faecalis</i>	Unidentified



**Table 3.4.1.** Growth response of BHRM<sup>a</sup> in presence of different heavy metals at different concentrations

Heavy metals	Conc. (ppm) <sup>b</sup>	Different genera of bacteria studied																
		<i>Pseudomonas</i>					<i>Proteus</i>					<i>Alcaligenes faecalis</i>						
		GO03	GO04	GO05	GP11	GP12	GP07	GP09	GP10	GO01	GO02	GP06	GP14	GP15	GP16	GP17	BI	B.sp
Mercury	25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mercury	50	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-
Mercury	55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mercury	75@	+	+	-	-	+	NT	NT	NT	NT	NT	NT	+	+	NT	NT	+	-
Cadmium	100	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
Copper	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Zinc	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lead	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

<sup>a</sup>in this study, all strains capable of tolerating 25 ppm Hg are considered as bacteria highly resistant to Mercury : BHRM;

<sup>b</sup>parts per million ( $\mu\text{g. ml}^{-1}$ ) spiked concentrations; +, positive growth; -, no growth; NT, not tested; PS, *Pseudomonas*; PR, *Proteus*; AF, *Alcaligenes faecalis*; BI, *Bacillus iodinium*; B.sp., *Bacillus sp.*; @in SWNA; in all other cases it was in SWNB.

**Table 3.4.2.** Growth response of MRB<sup>a</sup> in the presence of various xenobiotics tested at the concentrations in ppm mentioned against them

Code	Conc (ppm)	Different genera of bacteria studied																
		<i>Pseudomonas</i>				<i>Proteus</i>				<i>Alcaligenes faecalis</i>				B.sp BI				
		GO03	GO04	GO05	GP11	GP12	GP07	GP09	GP10	GO01	GO02	GP06	GP14	GP15	GP16	GP17	GP13	GP08
DDT	100	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	-	-
Penconazole	93	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-
Propiconazole	95	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Metolachlor	95	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pretilachlor	96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Profenofos	91	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenol	50	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Phenol	1000	NT	+	NT	NT	NT	NT	+	NT	NT	+	NT	NT	NT	-	NT	+	NT
Phenanthrene	UN	+	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
Benzene	UN	+	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
Crude oil	UN	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
TCE	10%	-	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	NT	+	NT	NT	NT

<sup>a</sup>in this study, all strains capable of tolerating 25 ppm Hg are considered as BHRM; <sup>b</sup>parts per million ( $\mu\text{g. ml}^{-1}$ ) spiked concentrations; +, positive growth; -, no growth; NT, not tested; UN, undefined; PS, *Pseudomonas*; PR, *Proteus*; AF, *Alcaligenes faecalis*; BI, *Bacillus iodinium*; B.sp., *Bacillus* sp.

**Table 3.4.3.** Growth response of MRB to different combinations of toxic substances

Bacterial genera	Combination of toxic substances			
	M2*+D13+P10 (25ppm)**	M5 + D10 + P10 (25 ppm)	M10+D5+P10 (25ppm)	M20+D10+P20 (50 ppm)
<i>Pseudomonas</i> (5)***	+	+	+	-
<i>Proteus</i> (3)	+	+	+	-
<i>Alcaligenes faecalis</i> (7)	+	+	+	-
<i>Brevibacterium iodinium</i> (1)	+	+	+	+
<i>Bacillus</i> sp. (1)	+	+	+	-

\*individual concentration (in ppm) of M, mercury; D, DDT; P, phenol in each combination;

\*\*cumulative concentration (in ppm) of M, D and P. For example, M2 means 2ppm Hg, D13, 13 ppm DDT and P10, 10 ppm phenol; \*\*\*number of strains tested; +, growth; -, no growth.

**Table 3.5.** Response of bacteria highly resistant to mercury to antibiotics in terms of inhibition zone in mm diameter

Antibiotics	Conc. (µg/disc)	Sensitivity limit	Isolates of BHRM						Reference culture
			CH03	CH07	CH12	GP06	GP15	GP16	<i>E. coli</i>
Kanamycin	30	18	12	22 (S)	10	17	16	11	20 (S)
Streptomycin	10	15	10	13	13	15	12	14	20 (S)
Neomycin	30	17	16	12	6	20 (S)	15	16	19 (S)
Tetracycline	30	19	6	7	8	11	9	6	21 (S)
Ampicillin	10	17	12	14	9	10	14	15	18 (S)
Chloramphenicol	10	18	12	19 (S)	16	17	13	14	29 (S)
Nalidixic acid	30	19	17	17	15	17	11	18	19 (S)
Gentamicin	10	15	21 (S)	22 (S)	18 (S)	25 (S)	12	16 (S)	28 (S)
Penicillin G	10 U	29	7	0	10	11	4	6	20

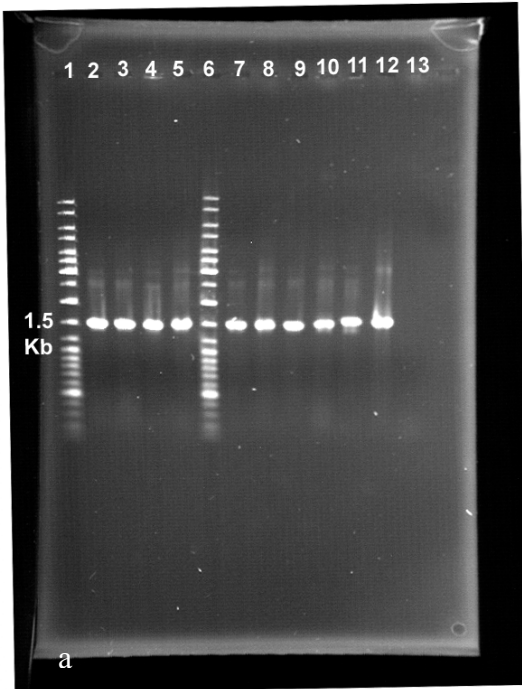
(S), sensitive; rests are resistant

**Table 3.6.** Carbon:nitrogen (C:N) ratios for different strains of MRB. These ratios are averages of three replicates.

Strains	C:N ratios in		
	SWNB without Hg	SWNB with 10 ppm Hg	SWNB with 50 ppm Hg
<i>P.aeruginosa</i> (CH07)	4.17	3.99	3.91
<i>A. faecalis</i> (GP15)	3.64	3.80	3.96
<i>A. faecalis</i> (GP16)	4.00	4.00	4.33

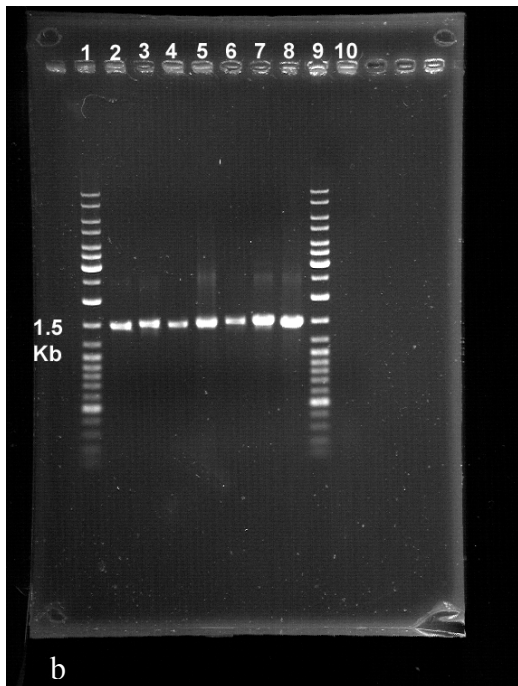
One-way ANOVA results for:

Isolate	$F_{1,5}$ ratio	$P$	variables
CH07	7.198	> 0.0551	0 and 10 ppm Hg
	11.868	< 0.0261	0 and 50 ppm Hg
GP15	39.767	> 0.0032	0 and 10 ppm Hg
	169.472	< 0.0002	0 and 50 ppm Hg
GP16	0.067	> 0.8088	0 and 10 ppm Hg
	453.457	> 0.00002	0 and 50 ppm Hg

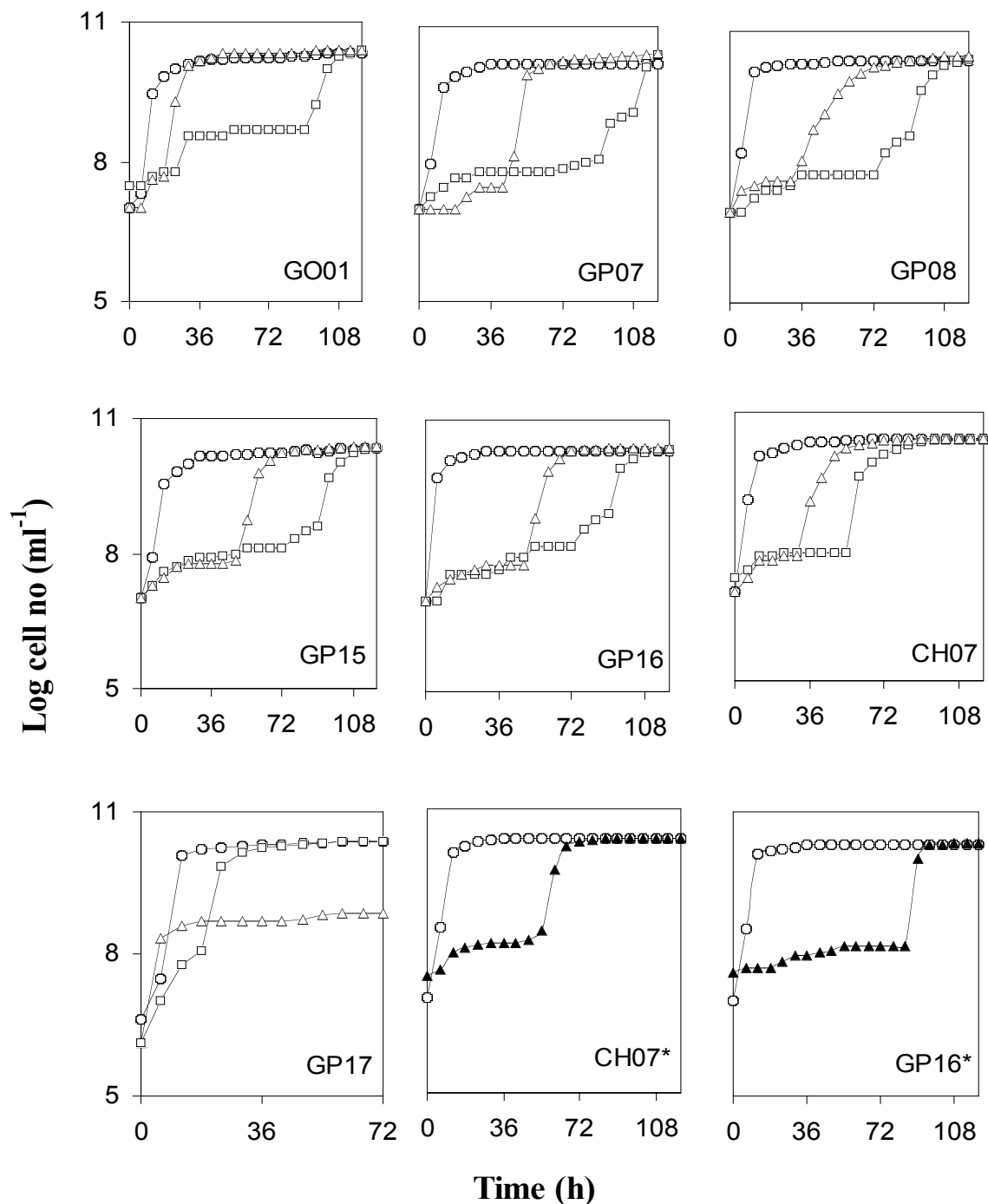


**Plates 3.1a & b.** 16S rDNA-PCR of MRB: Photograph of a gel with 16S rDNA fragments from different test cultures. The gel was electrophoresed through TAE buffer at 85 mV

**a.** Lanes (from left to right) 1, marker (100 bp ladder mix); lanes 2-13, GP08, 3C, GP13, GP16, CM10, 11c, *P. putida* KT2442 (+ve control), marker (100 bp ladder mix), PCR mix (-ve control); (3c & 11c are contaminants)



**b.** Lanes (from left to right): 1, marker (100 bp ladder mix), lanes 2-10, GO02, GP06, GP13, GP14, marker (100 bp ladder mix), GP15, GP16, GP17, CH07, CH13, *P. putida* KT2442 +ve control, PCR mix (-ve control)



**Figure 3.1.** Growth curves of different bacteria highly resistant to mercury (BHRM) in seawater nutrient broth (SWNB) with no added Hg (open circles); 10 ppm Hg (open triangles) and 50 ppm Hg (open squares). The isolates are *Pseudomonas aeruginosa* (CH07); *Alcaligenes faecalis* (GO01, GP15, GP16, GP17); *Proteus* (GP07); *Bacillus pumilus* (GP08). Growth curves of GP16 and CH07 isolates with asterisks represent the growth curves (filled triangles) when Hg (20 ppm), phenol 20 ppm and DDT 10 ppm were added together to SWNB to see the synergistic effect of these chemicals on MRB in terms of growth.

**APPENDIX :**

**Modified complex liquid medium\* :**

Filtered aged seawater-	500 ml
Distilled water-	500 ml
Tris-	6.055 g
K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O-	0.058 g
FeSO <sub>4</sub> -	0.003 g
Yeast extract-	3.0 g
Peptone-	5.0 g

\*the original composition had artificial seawater in place of seawater and 1.016 g NaCl.