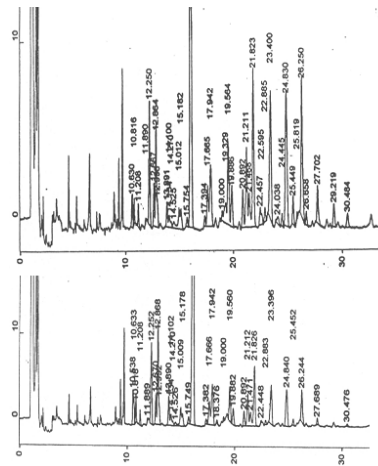
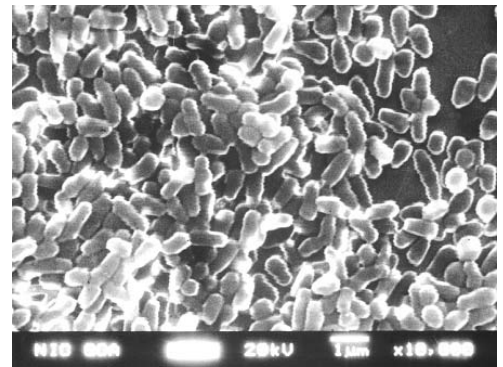
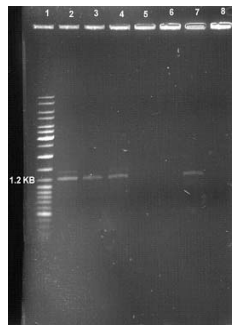
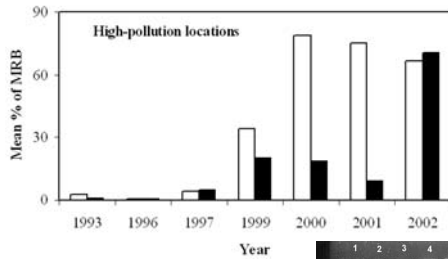


# Mercury-resistant marine bacteria and their role in bioremediation of certain toxicants

Thesis submitted to Goa University

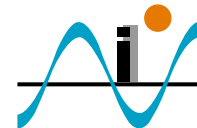
for the degree of

Doctor of Philosophy in Marine Sciences



Jaysankar De

National Institute of Oceanography  
Council of Scientific & Industrial Research  
Dona Paula, Goa - 403 004, INDIA



November 2004

**MERCURY-RESISTANT MARINE BACTERIA AND THEIR ROLE IN  
BIOREMEDIATION OF CERTAIN TOXICANTS**

**Jaysankar De**

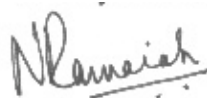
National Institute of Oceanography  
Dona Paula – 403 004, Goa, India

**November 2004**

## CERTIFICATE

This is to certify that **Mr. Jaysankar De** has duly completed the thesis entitled  
“**Mercury-resistant marine bacteria and their role in bioremediation of certain  
toxicants**” under my supervision for the award of the degree of Doctor of Philosophy.

This thesis being submitted to the Goa University, Taleigao Plateau, Goa for the award of  
the degree of Doctor of Philosophy in Marine Sciences, is an original record of the work  
carried out by the candidate himself and has not been submitted for the award of any  
other degree or diploma to this or any other University in India or abroad.



(N. RAMAIAH)

Research Guide

Scientist

Biological Oceanography Division

National Institute of Oceanography

Dona Paula, Goa-403004

Date: November 4, 2004

Place: NIO, Goa

## **DECLARATION**

I hereby declare that the thesis titled “Mercury-resistant marine bacteria and their role in bioremediation of certain toxicants” submitted by me is not the same as one which might have already been submitted for a degree or any other academic qualification to any University in any country.

Date: November 4, 2004

Place: NIO, Goa



(DE JAYSANKAR)

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## ABBREVIATIONS USED IN THIS THESIS

AAS= Atomic absorption spectrophotometer  
ASN= Artificial seawater nutrient  
ASW= Artificial seawater  
BHRM= Bacteria highly resistant to mercury  
CFU= Colony forming unit  
C:N= Carbon:Nitrogen  
CHN= Carbon hydrogen nitrogen  
CPD= Critical point drying  
DNA= Deoxyribonucleic acid  
dNTP=deoxynucleoside tri phosphate  
ECD= Electron capture detector  
EDS= Energy dispersive x-ray spectrometry  
EDTA= Ehtylene diamine tetra acetic acid  
EtBr= Ethidium bromide  
FAD= Flavo adenine dinucleotide  
FIAS= Flow injection atomic spectrometry  
FID= Flame ionization detector  
GC= Gas chromatography  
ICP-AES= Inductively coupled plasma – atomic emission spectrometry  
LB= Luria Bertani  
MB= Marine broth  
MBT= Monobutyltin  
MRB= Mercury-resistant bacteria  
MSM= Minimal salt medium  
NADP= Nicotin amide adenine dinucleotide phosphate  
NADPH= Nicotin amide adenine dinucleotide hydrogen phosphate  
OD= Optical density  
PAGE= Poly acrylamide gel electrophoresis  
PBS= Phosphate buffer saline  
PCBs= Polychlorinated biphenyls  
POPs= Persistent organic pollutants  
PPB= Parts per billion ( $\equiv \mu\text{g l}^{-1}$ )  
PPM= Parts per million ( $\equiv \text{mg l}^{-1}$ )  
PPT= Parts per trillion ( $\equiv \text{ng l}^{-1}$ )  
RNA= Ribonucleic acid  
SDS= Sodium dodecyl sulphate  
SEM= Scanning electron microscopy  
SWNA= Seawater nutrient agar  
SWNB= Seawater nutrient broth  
TAE= Tris acetic acid EDTA  
TBT= Tri butyl tin



*Dedicated To My  
Beloved Parents*

## 1.1. INTRODUCTION:

Microbial world is invisible to unaided human eyes. Thus what goes on in their domain is not easily perceived, unless some direct measurements and analyses are carried out. Comprised of bacteria, fungi, yeasts, phytoplankton, protozoans and other microfauna within 500  $\mu\text{m}$  size, microbial communities perform immense tasks mainly to keep themselves perpetuating in their ecosystems. Though it is for their survival, growth and reproduction, the microbial activities of photosynthesis (by microscopic phytoplankton), respiration by all living beings) and breaking down of organic matters into simpler moieties and finally to inorganic molecules by all heterotrophic beings are the pivotal roles that help the earth's ecosystems function and attain, as far as possible a dynamic equilibrium. On the whole sunlight is the only external input the life on earth needs. All other matter is produced, consumed and recycled by an array of organisms inhabiting the earth in her varied and often extreme habitats. In essence, the environmental functioning and stability are continuously aided and maintained by microscopic organismic activities. Any imbalance largely due to their activities through human and natural effects adversely affects the ecosystems. Pollution due to anthropogenic activities is the greatest problem all the ecosystems were subjected to right from the beginning of human dominance through the hunting-gathering, settled agricultural to modern industrialized civilizations. In the last two millennia or so, the human population growth and indiscrete uses of earth's non-renewable resources have brought about rapid changes to the extent that there are already innumerable degraded/retarded habitats spoiling the Mother Earth rather undesirably. The long term consequences of retarded habitats are too numerous including shifts in human settlements, societal conflicts, diseases, economic losses, community and species diversity shifts, global changes and health effects. The one "adjustment" natural organismic community makes in the

face of all and life-threatening ill effects of pollution in their metabolic potential is modification in its life-style so much so that some of its representatives “go-on”. This adjustment can be termed variously as tolerance or resistance. While many components of organismic communities have the potential for such adjustment, the focus of this thesis will be the bacterial resistance. As, in industrial areas both metal and organic pollution is of vital concern, interest in bacterial resistance to metal salts, especially when associated with degradative activities, is of practical significance. Though studies on tolerance to toxic metals and degradative capacities have been extensive, these two aspects are often addressed separately. Often they have not taken into account that in industrial areas organic pollution (fossil fuels or their derivatives, pesticides, PCBs and TBT among others) is often accompanied by inorganic ones mainly of heavy metals (mercury, cadmium, lead to name a few). Without efficient retention technologies, toxic chemicals including Hg are let into the environment, endangering ecosystems and public health.

The main focus in this section is on literature review on mercury-resistant marine bacteria, mercury bioremediation and potential of mercury-resistant bacteria (MRB) in detoxifying /degrading other toxic chemicals. The review will also be extended to Hg, Cd, Pb, PCBs and TBT as the bacterial potential to biodegrade/biotransform/bioremediate these toxicants were examined rather in greater details during this study. While the detailed literature review along with certain interpretations will be available under different chapters of this thesis, this introductory effort is to provide broad views on the past researches on each of the specific toxicants examined in this study.

Mercury resistant bacteria (MRB) are those bacteria that grow in presence of 10 ppm ( $\text{mg l}^{-1}$ ) mercury (as  $\text{HgCl}_2$ ) in the seawater nutrient agar medium (SWNA). Izaki (1981) had used 2 ppm

mercury (as  $\text{HgCl}_2$ ) to isolate mercury-resistant bacteria whereas Baldi et al. (1989) used 10 ppm Hg for the same. Many of these MRB were able to grow at an Hg (as  $\text{HgCl}_2$ ) concentrations of 25 ppm or higher and were termed as bacteria highly resistant to mercury (BHRM; De et al., 2003).

## **1.2. Bacterial bioremediation of mercury contamination:**

### **1.2.1. Mercury in the environment:**

Mercury, the only metal in liquid form at room temperature is the most toxic of the heavy metals (Gerlach, 1981) and the sixth most toxic chemical in the list of hazardous compounds (Nascimento & Chartone-Souza, 2003) has been present in the environment for aeons. Erupted from the core of earth by volcanic activity it exists as mineral (mostly as cinnabar- $\text{HgS}$ ), as mercuric oxide, oxychloride, sulfate mineral (Kim et al., 2001) or also as elemental mercury. It also exists as gas due to its high vapour pressure. In a biogeochemical cycle (Figure 1.1) mercury is globally dispersed undergoing many physical and chemical transformations (Barkay, 2000; Barkay et al., 2003):

1. in the atmosphere elemental mercury is photo-oxidized to ionic mercury ( $\text{Hg}^{2+}$ ).
2. rain precipitates the inorganic mercury on the surface of the earth, where carried out mainly by microorganisms in aquatic systems,
3. it is reduced back to its elemental form or
4. methylated.
5. elemental mercury evaporates into air where the cycle begins anew.

The four main natural processes that aid Hg emission are:

1. degassing from geological mineral deposits,
2. emissions from volcanic activities,

3. photoreduction of divalent mercury in aquatic systems and
4. biological formation of elemental and methyl mercury.

Although it is undisputed that mercury occurs naturally and toxic concentrations in some locations, mercury emissions owing to anthropogenic activities (mainly through chloralkali-electrolysis and chlorine production), mining and fossil fuel combustion or waste incineration are immense, contributing substantially to the mercury pool participating in the biogeochemical cycle (Figure 1.1). However, the concentrations of mercury in various compartments from natural and anthropogenic sources are highly variable. Recent measurements of mercury in aquatic systems have given the following concentration ranges: open ocean ( $0.5\text{-}3\text{ ng l}^{-1}$ ), coastal seawater ( $2\text{-}15\text{ ng l}^{-1}$ ), rivers and lakes ( $1\text{-}3\text{ ng l}^{-1}$ ). These may be considered representative for dissolved mercury <http://www.inchem.org/documents/ehc/ehc/ehc086.htm#SectionNumber:3.3>). Recently, the stringent legislation in the US or European countries have brought down the anthropogenic input and the global mercury estimate is on the decrease (Slemr, 2003). Mercury is widely used in industry because of its diverse properties like it is odorless, liquid at room temperature, tolerates high temperature and is highly volatile in nature.

Worldwide many areas contaminated with mercury pose threat to people and environment (Xiao et al., 1998; Cleckner et al., 1999; Fukuda et al., 1999; Horvat et al., 1999). Owing to the mercury cycle described earlier, regional emissions may be deposited elsewhere, in far off uninhabited regimes, for e.g., in the Arctic (Macdonald et al., 2000). Hence local, regional and small-scale contaminations do not remain confined to a particular area but affect ecosystems globally. Mercury (mostly as methylmercury) accumulates in carnivorous fish via the food chain by a process called biomagnification and poison people consuming fish. The syndromes such as neurological disorders, resulting from the poisoning have been named Minamata disease in the

late 1950s when over 3000 people around this Minamata Bay in Japan were severely poisoned by methylmercury pollution caused by a chemical manufacturing plant releasing its effluents containing mercury (Langford & Ferner, 1999).

### **1.2.2. Toxicity of mercury:**

Mercury was widely used in clinical thermometers before the prohibition by the European Union directive (European Council Directive 93/42/EEC). Moreover, it can be found in several medications and is still used in dental fillings. However, its toxicity has also been known and recorded for two millennia (Langford & Ferner 1999). Different forms of mercury possess different characteristics rendering it a hazard to living organisms. The toxicity of highly reactive mercuric mercury ( $\text{Hg}^{2+}$ ) is attributed to its binding to the sulphhydryl groups, of the cysteines of essential enzymes and proteins, thus disturbing vital cell functions. In the body water-soluble ionic mercury salts are not efficiently absorbed. Rather, they are eliminated from the body via the kidneys acutely causing damage to the gut and the renal systems. The hazard arising from elemental mercury ( $\text{Hg}^0$ ) is owed to its high vapour pressure allowing it to be easily inhaled. Absorbed by the lungs it enters the blood and circulated throughout the body including the brain. Elemental mercury is transformed in the red blood cells (RBC), the liver and central nervous system (CNS) to  $\text{Hg}^{2+}$  and methylmercury. Repeated or prolonged exposure mainly results in vasomotor disturbances, tremor and behavioural disturbances. Organic mercury compounds like mono or di methylmercury or phenylmercuric acetate (PMA) are lipid soluble and thus readily absorbed into the body. They penetrate membranes and cross the blood-brain barrier. A large proportion of organic mercury is transformed into reactive  $\text{Hg}^{2+}$  (Strasdeit, 1998) and can severely damage the CNS causing neuromuscular malfunctions, ranging from numb limbs, visual disorders to paralysis and even death (Barkay 2000). The clinical and epidemiological evidences

indicate that the prenatal life is more sensitive to toxic effects of methylmercury than adults which results into abnormal neuronal development, leading to altered brain architecture and brain size. Because transformation to  $\text{Hg}^{2+}$  occurs slowly, symptoms of poisoning with organic mercury may appear weeks or months later to the initial poisoning as was detected in the case of Karen Wetterhahn, a chemistry professor (Nierenberg et al., 1998).

Today, the major source of human exposure to mercury is the consumption of seafood (Boudou & Ribeyre, 1997). Mercury compounds are acutely toxic to freshwater microorganisms. The no-observed-toxic-effect-level (NOTEL) for inorganic mercury lies between 1-50 ppb ( $\mu\text{g l}^{-1}$ ), depending on the organism, density of the cell in culture, and experimental conditions. Diversity of species in a mixed culture may be affected by 40 ppb  $\text{HgCl}_2$  (Canstein, 2000). For organic mercury, the NOTEL is 10-100 times lower. Aquatic plants sustain damage after exposure to inorganic mercury at concentrations of 800-1200 ppb. Organomercury exerts toxicity at concentrations 10-100 times lower. Many aquatic invertebrates, particularly larvae are sensitive to mercury toxicity. For the most sensitive species, *Daphnia magna*, the NOTEL for reproductive impairment is 3 ppb for inorganic mercury and lesser than 0.04 ppb for methylmercury (Canstein, 2000). Hence it is of great importance for both environment and public health to avoid mercury contamination/discharges into freshwater and marine ecosystem.

### **1.2.3. Bacterial resistance to mercury:**

As a response to toxic mercury compounds globally distributed by geological and anthropogenic activities, microbes have developed a surprising array of resistance mechanisms to overcome Hg toxicity. An extensively studied resistance system based on clustered genes in an operon (i.e.

*mer*), allows bacteria to detoxify  $\text{Hg}^{2+}$  into volatile mercury by enzymatic reduction (Komura & Izaki, 1971; Summers, 1986; Misra, 1992; Silver, 1996; Osborn et al., 1997; Barkay et al., 2003).

It appears that bacterial resistance to mercury is an ancient mechanism, probably acquired even before anthropogenic usage of mercury. Since the same biotransformations that constitute the Hg biogeochemical cycle can take place inside the human body, understanding its external transformations and transport processes will help in figuring out which of these processes can exacerbate or ameliorate Hg toxicity in humans (Barkay et al., 2003).

Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. They vary in number and identity of genes involved and are encoded by the *mer* operon located on plasmid (Summers & Silver, 1978; Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994; Osborn et al., 1997), chromosomes (Wang et al., 1987, 1989; Inoue et al., 1989, 1999; Iohara et al., 2001), transposons (Misra et al., 1984; Kholodi et al., 1993; Liebert et al., 1997, 1999; Hobman et al., 1994) and integrons (Liebert et al., 1999).

Mercuric mercury complexes with organic and inorganic ligands and, is easily adsorbed to surfaces of particulates owing to its high reactivity and affinity to thiol groups (Barkay, 2000). Mercury bioavailability therefore plays a crucial role in the evaluation of microbial resistance levels. There is some tolerance towards mercury owing solely to unspecific sequestration by cell walls and lipopolysaccharide (LPS) layers (Langley & Beveridge, 1999).



The narrow mercury resistance operon (*mer*) comprises three major functions: transport of  $\text{Hg}^{2+}$  into the cell, enzymatic NADPH-dependent conversion of the ionic mercury into relatively less toxic elemental mercury ( $\text{Hg}^0$ ) and regulation of the functional genes (Misra, 1992; Silver & Phung, 1996). Detoxification of organic mercury requires a fourth function, namely the cleavage of mercury from the organic residue and the resistance is termed as “broad spectrum”. Genes conferring these functions are designated as *merT*, *merP* (transport), *merA* (mercury reduction), *merB* (cleavage of Hg from organic residue), *merR* and *merD* (regulation). Further, more of *mer* genes have been identified recently; *merC* and *merF*, both membrane proteins, conferring transport functions (Wilson et al., 2000). Also *merG* is known to confer resistance to phenylmercury (Kiyono & Pan Hou, 1999). Most mercury resistance operons are inducible, i.e.  $\text{Hg}^{2+}$  has to be present in order to activate expression of resistance whereas transcription is suppressed in the absence of mercury (Misra, 1992). If, however, switched on by mercury at a contaminated site, the bacterial mercury resistance forms the basis of natural on-site detoxification (Silver et al., 1994) under aerobic conditions. Mercury pollution can contribute to increased antibiotic resistance (McArthur & Tuckfield, 2000). The combined expression of resistance to antibiotic and mercury may be caused by selection, as a consequence of the mercury present in an environment (Sant’ana et al., 1989).

Many researchers have claimed that Hg-reductase activity in Hg-resistant bacteria is always inducible and never constitutive (Summers, 1986). However, Nakamura et al. (1986) showed the presence of a constitutive Hg-reductase in *Streptomyces* sp. Induction of the *mer* operon has been proposed to take place when the concentration of mercury exceeds 50 pM ( $\mu\text{10 ppt}$ ), microbial reduction via *merA* reductase likely becomes the predominant mechanism of Hg (II) detoxification (Morel et al., 1998).

#### **1.2.4. Mercury-resistant bacteria in bioremediation:**

The ability of bacteria to detoxify mercury can be utilized to bioremediate mercury contaminated wastewater. In fact, it seems as if this potential was solely confined to microbial species as no naturally occurring plant or animal species have been reported to detoxify mercury. Nevertheless, plants have been engineered to overexpress the bacterial mercury resistance and transform organic and inorganic mercury to elemental mercury with promising result for phytoremediation of mercury-contaminated sites (Rugh et al., 1998; Bizily, 1999). Plants combine many practical benefits for the bioremediation of contaminated sites. With the mercury transforming transgenic yellow poplar (Rugh et al., 1998), large areas of contamination could be dealt with. In addition to decontamination, these with stout-root trees would stabilize the soil surface and provide niches for the inhabitation of mercury- reducing microbes. However, the mercury reducing plants emit volatile  $\text{Hg}^0$  in to the air, which although non-significant, on a global scale, may contribute to increased mercury concentrations locally.

One of the initial efforts to retain mercury in bacterial bioreactors was made by Brunke et al. (1993). They managed to capture elemental mercury in globules upto 5  $\mu\text{m}$  in diameter in fixed-bed columns using genetically engineered mercury-reducing bacteria that were immobilized on ceramic carriers, glass or in alginate beads. Some years later, Canstein et al. (1999) demonstrated the removal of mercury from chloralkali electrolysis wastewater by a mercury resistant *Pseudomonas putida* strain. This natural isolate was capable of coping with upto 8 ppm of mercury in the wastewater, transforming >97% (when cellulose fibers were the carriers) or 98.5% (with siran as the carrier). These laboratory-scale reactor results formed the basis for the development of a technical scale bioreactor that decontaminated mercury-polluted chloralkali wastewater *in situ* (Wagner-Döbler et al., 2000, 2003). Mercury retention could be achieved upto

95% efficiency, and the discharge limit for mercury in industrial wastewater (50 ppb) could be met reliably with the help of an activated carbon filter at the end of the line, capturing residual traces of mercury. The elemental mercury accumulated in the bioreactor, this however, did not affect the efficacy of the bioreactor.

It was found that although inoculated with several mercury resistant isolates, foreign mercury-resistant bacteria invaded the reactor and a new consortium of mercury-transforming bacteria evolved that dynamically changed over time (Canstein et al., 2001). In experiments in the laboratory-scale bioreactors, the presence of a consortium could be shown to be of benefit for a reliable, disturbance-independent mercury-removal (Canstein et al., 2002). Due to the continuous selective pressure in the bioreactor, MRB that are most properly adapted to onsite conditions are enriched and this is best suited to improve bioreactor stability.

### **1.3. Other heavy metals:**

#### **1.3.1. Cadmium:**

After mercury, cadmium (Cd) is the next of the most toxic heavy metals and has already caused several environmental problems including the most painful affliction, *itai itai* (Nogawa & Ishizaki, 1979; Matsuda et al., 2003). Its reported concentration ranges average  $0.1 \mu\text{g liter}^{-1}$  or less in seawater, less than  $0.01$  to  $0.06 \mu\text{g liter}^{-1}$  in unpolluted fresh waters and up to  $5 \text{ mg kg}^{-1}$  and  $0.03$  to  $1 \text{ mg kg}^{-1}$  in inland and marine sediments, respectively. To eliminate environmental effects, emissions of cadmium should be reduced from smelters, incinerators, sewage sludge applied to the land, phosphate fertilizers, Cd-containing manures. ([http://arcweb.sos.state.or.us/rules/0103\\_Bulletin/0103\\_ch603\\_bulletin.html](http://arcweb.sos.state.or.us/rules/0103_Bulletin/0103_ch603_bulletin.html)). Microbial flora

detoxifying Cd is of pertinence in dealing with Cd pollution. In this regard, studies by Fein & Delea (1999); Bang et al. (2000); Roane et al. (2001); Essa et al. (2002); Lebeau et al. (2002) have shown the potential of Cd detoxification/ biotransformation.

### **1.3.2. Lead:**

The major sources of lead in the environment, of significance to living organisms, arise from lead mining, and from refining and smelting of lead and other metals. The major dispersive, non-recoverable use of Pb is in the manufacture and application of alkyl lead fuel additives. The concentration of lead in seawater ( $0.03\text{-}0.4 \mu\text{g liter}^{-1}$ ) has been found to be lower than in freshwaters. Lead is well known to inhibit the biosynthesis of heme, and consequently of hemoglobin and to decrease the life span of circulating red blood cells (Potula, 1996). Once thought to be safe, even at low concentration Pb leads to decreased IQ scores, slow growth, hearing problems and kidney damage. Accidental exposure of pregnant women can result in premature birth, low birth weight, neurological deficits or even abortion. Several studies (Chen et al., 1995; Chang et al., 1997; Roane, 1999; Slaveykova & Wilkinson, 2002) have been carried out on bioremediation of Pb.

## **1.4. Xenobiotics:**

### **1.4.1. PCBs:**

Poly-chlorinated biphenyls (PCBs) are among the most persistent organic pollutants (POPs). Globally, PCBs are found in air concentrations of  $0.002$  to  $15 \text{ ng m}^{-3}$ . In industrial areas, levels are higher (up to  $\mu\text{g m}^{-3}$ ). In rainwater and snow, PCBs are found in the range of  $1 - 250 \text{ ng l}^{-1}$ . Surface water may be contaminated by PCBs from atmospheric fallout, from direct emissions

from point sources, or from waste disposal. Under certain conditions, levels of up to 100-500 ng l<sup>-1</sup> in water have been measured. In the oceans, levels of 0.05-0.6 ng l<sup>-1</sup> have been found (<http://www.inchem.org/documents/ehc/ehc/ehc140.htm>). The usage of these POPs has been banned from early 1990s (Wiegel and Wu, 2000). Since they persist, get dispersed over to very vast areas and their estimated half-life in the environment is over a few months, concerted efforts must be made to cleanup PCB contaminated sites, electrical utilities and other places. Investigations by Bedard et al. (1987), Kimbara et al. (1999), Yadav et al. (2000) and Sarkar et al. (2003) have shown that a few bacterial species can detoxify many congeners of the 209 known PCBs.

#### **1.4.2. TBT:**

After Tributyltin (TBT) had been used widely for its antifouling property since 1970, most of the European countries, North America and Hong Kong imposed regulation on its usage in 1980s (Dowson et al., 1993; de Mora, 1996; Minchin et al., 1997; Evans, 1999, Champ, 2000, 2001). TBT was banned way back in 1942 in France (Alzieu et al., 1986, 1989). The International Maritime Organization (IMO) has already passed the resolution to ban on use of TBT-based antifouling compounds (Champ, 2000). It has been shown that TBT may be responsible for thickening of oyster and mussel shells as well retardation of growth in aquatic snails (Alizeiu & Heral, 1984; Laughlin et al., 1986). Recent estimate shows that the annual world production of organotins may be close to 50,000 tons yr<sup>-1</sup> (Inoue et al., 2000). Non-point source of environmental exposure include discard and sanitary landfill, disposal of plastic and direct release of biocides into freshwater or marine environment. TBT degradation by photolysis alone proceeds slowly with a half-life of >89 days (Wuertz et al., 1991). In aquatic systems, both pH and salinity determine organotin speciation and therefore activity (White et al., 1999). However,

as with other inorganic and inorganic forms of toxic metals, many microorganisms may exhibit organotin resistance or tolerance, with some examples of organotin biodegradation being reported (Cooney & Wuertz, 1989; Cooney, 1995). The inhibition of microbial processes by tributyltin has been recorded for all major groups, with the main interactions occurring at cellular membranes and chloroplasts, or the mitochondria in eukaryotes (Gadd, 2000). TBT was considered less degraded by the bacteria in sediment than in water (Barug, 1981; Stewart & Mora, 1990; Dowson et al., 1993). However, experiments on degradation of TBT in pure culture conditions have been few (Kawai, 1998; Dubey & Roy, 2003).

#### **1.5. Bacterial resistance to multiple toxicants:**

*Klebsiella pneumoniae*, a mercury-resistant bacterial strain has been reported to possess high tolerance to heavy metals such as mercury 2400  $\mu\text{M}$ , lead 8000 $\mu\text{M}$ , silver 2400  $\mu\text{M}$  and cadmium 1000  $\mu\text{M}$  (Zeroual et al., 2001). Essa et al. (2002) reported that *Klebsiella pneumoniae* M426 possesses three different mechanisms to detoxify mercury. These are (1) *merA* mediated volatilization, (2) aerobic assimilatory sulphate reduction producing  $\text{H}_2\text{S}$  and thereby  $\text{HgS}$  and (3) biomineralization of  $\text{Hg}^{2+}$  as an insoluble mercury-sulphur complex other than  $\text{HgS}$ . Interestingly enough, this same bacterium was able to aerobically precipitate toxic cadmium as cadmium sulfide. Pain et al. (1998) reported that most of the TBT-resistant bacteria are also resistant to six heavy metals (Hg, Cd, Zn, Sn, Cu, Pb), which suggest that resistance to these kinds of toxic chemicals may be present in the same organism. Usually, TBTC tolerant strains show cross-tolerance to methylmercury (Suzuki et al., 1992). Fukagawa et al. (1994) reported 11 bacterial isolates that were resistant to organotin and methylmercury and the genes conferring resistance to organotin might be present on the chromosomes (Fukagawa et al., 1993; Suzuki et al., 1994).

## **1.6. Overview of this Thesis:**

The adaptability of natural microbial communities in pollution-affected environments is an important requirement in their survival and ecological performance. Monitoring of bacterial responses (and 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). 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, 1998, 2001) and to realize the potential of such flora in detoxifying toxic substances (Barbieri et al., 1996; Canstein et al., 1999). Hazardous chemicals present in the environments exert selection pressure and thereby permit the resistant flora/fauna that will work towards voiding off unhealthy conditions due to toxic chemicals continue to persist. Long-term exposure to heavy metals (Zn, Cu and Ni) (Bååth et al., 1998) and Hg (Müller et al., 2001b; Ramaiah & De, 2003) has been found to alter the microbial community. Ecological analyses for quantifying MRB are presented in the **chapter 2** of this thesis.

Mercury-resistant bacteria are extremely important in detoxifying the mercury compounds by two separate enzymes acting sequentially namely, organomercurial lyase that cleaves the carbon-mercury bonds of certain organomercurials and mercuric reductase, which reduces  $\text{Hg}^{2+}$  to volatile mercury (Nakamura et al., 1990). A few studies (Barbieri et al., 1989, 1996; Ka et al., 1994) have examined marine mercury-resistant bacteria (MRB) and their potential to catabolize toxic xenobiotics. During this study, 30 bacterial isolates from marine samples were examined for their biochemical characteristics. They were subjected to morphological, physiological and nutritional characterization using different physical and biochemical tests following MacFaddin (1980). Thirteen of these isolates representing different groups were subjected to 16S rDNA

sequencing. In addition, the effect of Hg on carbon:nitrogen ratio was also examined to realize whether such basic physiological alterations are brought about by mercury. **Chapter 3** documents the results from this investigation.

Although much is known about the genetics of mercury resistance and detoxification by bacteria under laboratory conditions, the molecular basis for mechanisms that lead to development of mercury tolerance and adaptation to high or episodic concentrations of mercury in natural microbial communities are yet unclear (Ogunseitan, 2002). Molecular basis of mercury-resistance in MRB isolated from marine environs of India were examined and the details are furnished in **Chapter 4**. Genetic and biochemical studies have advanced our knowledge, leading to in-depth understanding of the resistance mechanism, gene evolution, and regulation of expression of the *mer* genes (Misra, 1992). Elevated levels of mercury exclusively select bacteria tolerating such levels (Barkay & Olson, 1986; Nakamura et al., 1986, 1988a, 1998b; Osborn et al., 1993). Resistance against highly toxic mercury compounds, mediated by the microbial *mer* operon was discovered by Summers & Lewis (1973) and, since then, several “archaetypical” *mer* operons (Brown et al., 1991; Liebert et al., 1999) have been studied with respect to structure, function and regulation of individual gene products (Ji & Silver, 1995; Nies, 1999; Barkay et al., 2003). Sequence analyses from some of these isolates revealed that the *mer* operon is a genetic mosaic with a novel modular arrangement (Summers, 1986) of essential genes and accessory genes. Some of the molecular aspects of *mer* genes in a select set of marine isolates are also furnished in **chapter 4**.

Perhaps the most difficult and inherent problem in metal remediation is that metals released during catabolic processing of metal-containing compounds are not degradable in the same sense



as carbon-based molecules. As is well known, the toxic metal atom is not useful as building block for new cellular components, and while a significant amount of carbon is released during catabolism to the atmosphere as CO<sub>2</sub>, while the metal may often not be volatilized. Thus, incorporation into the cell mass and volatilization facilitate carbon removal from environmental systems. Unless removed completely either through precipitation, ligand binding or effused out in gaseous form from a system, all biologically non-essential metals will persist indefinitely (Roane & Kellogg, 1996). The removal rates of Hg, Cd, and Pb by marine MRB was studied using a few BHRM and these findings are presented in **chapter 5**.

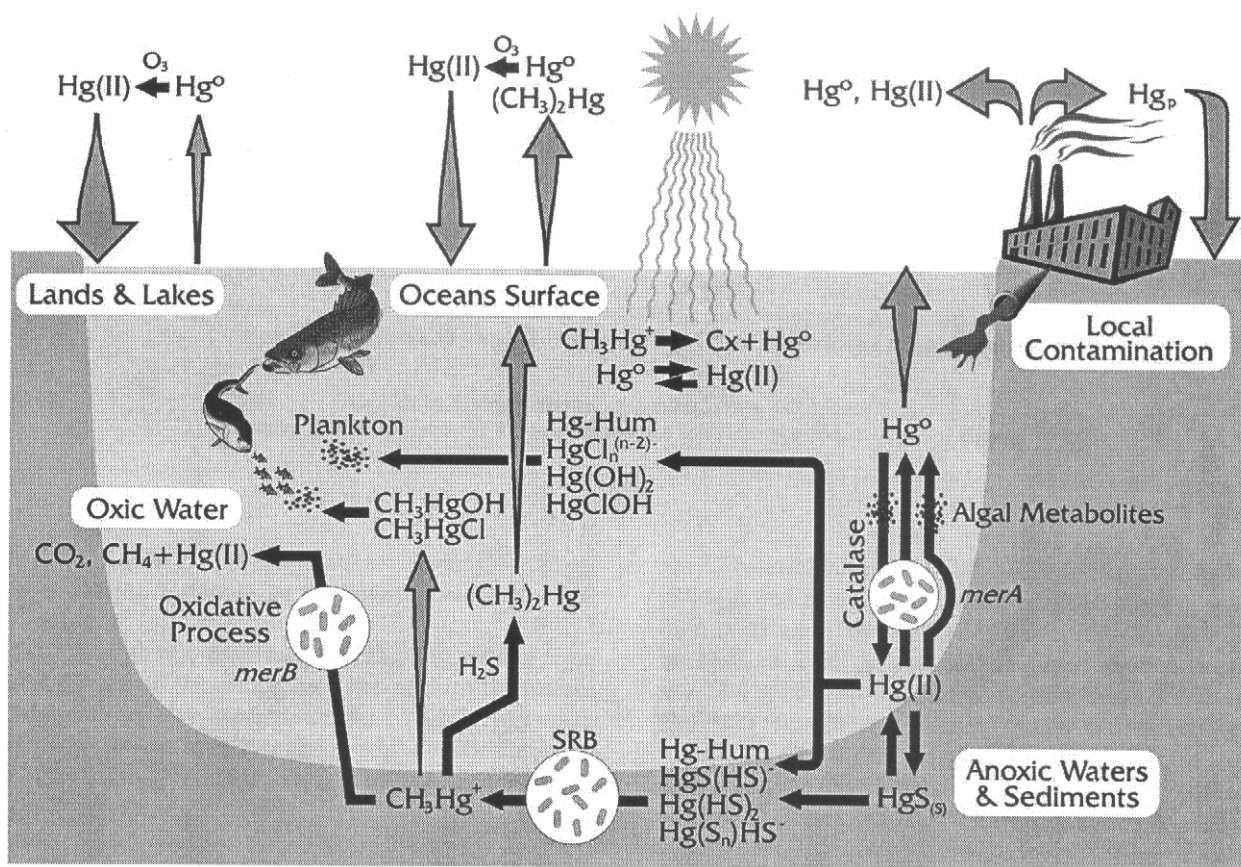
Biodegradation is nature's way of recycling wastes, or breaking down organic matter into nutrients that can be used by other organisms. By harnessing these natural forces of biodegradation, we can reduce wastes and clean up some types of environmental contaminants. Wastewater treatment also accelerates natural forces of biodegradation. A common approach to investigate biodegradation potential for recalcitrant pollutants such as PCBs, TBT and high molecular weight polynuclear aromatic hydrocarbons (PAHs) has been to isolate degrading strains using selective enrichment with more easily degraded analogues. The highly deleterious ecological and human effects of PCBs began to be realized only lately in the mid-eighties and the usage of PCBs has been banned (Wiegel & Wu, 2000). The use of indigenous microflora in biotreatment has been successfully employed for hydrocarbon remediation (Sherman, 1990; Atlas, 1991). Viney & Bewley (1990) tested the effect of surfactants on PCB degradation. Furukawa (2000) recently suggested studying PCB-degradation as a model system in particular to deal with the detoxification of POPs and, towards better understanding of xenobiotic degradation in general.

One of the most widely used marine paint amalgamations is Tributyltin, TBT (Dowson et al., 1993; de Mora, 1996; Minchin et al., 1997; Evans, 1999; Champ, 2000, 2001). This is mainly because of its potent toxicity to most marine flora and fauna (Gadd, 2000; Dubey & Roy, 2003) at its very low concentrations. As biofouling on the submerged surfaces coated with TBT fortified paints is drastically reduced, it is a great advantage to the shipping industry in terms of cost cuts. However, as with other organic and inorganic forms of toxic metals, many microorganisms do exhibit organotin resistance or tolerance, with a few examples of organotin biodegradation being reported (Cooney & Wuertz, 1989; Cooney, 1995). As degradation of TBT in pure culture conditions has not been widely investigated (Kawai, 1998; Dubey & Roy, 2003), experiments on degradation of PCBs and TBT by marine MRB were conducted and their results are described in **chapter 6**.

Bioremediation provides a good cleanup strategy for some types of pollution, although not at all contaminated sites/situations. Depending on the site and its contaminants, bioremediation may be safer and less expensive than alternative solutions such as incineration or landfilling of the contaminated materials. Bioremediation through an application of bacterial-resistance is quite cost-effective in comparison with chemical methods of mercury-decontamination such as sulfide precipitation or ion exchange columns. All it requires in addition to bacteria is a feeding solution (sucrose/glucose/others) from which bacteria can derive their energy required for reduction of mercury. In this regard, microbial-remediation has proved quite promising for its removal. Some of the laboratory-scale reactor results from Brenner et al. (1994), Brunke et al. (1993) and Canstein et al. (1999) formed the basis for the development of a technical scale bioreactor that decontaminated mercury-polluted chloralkali wastewater *in situ* (Wagner-Döbler et al., 2000, 2003). The potential of MRB to detoxify Hg from growth medium (F2) for cyanobacteria and for

a salt-tolerant rice was studied in order to realize the effective bacterial bioremediation achievable. These details are described in **chapter 7**.

In **chapter 8** all the researches carried out for this thesis work are briefly summarized with salient conclusions and certain suggestions for the future work.



**Figure 1.1.** The biogeochemical cycle of mercury in the environment (Barkay et al., 2003). Solid arrows represent transformation or uptake of mercury. Hollow arrows indicate flux of mercury between different compartments in the environments. The width of the arrows is approximately proportional to the relative importance of the flux in nature. Speciation of Hg(II) in oxic and anoxic waters is controlled by chloride and hydroxide, and by sulfide respectively. Circles depicting bacterial cells represent transformations known to be mediated by microorganisms. A group of dots indicate the involvement of unicellular algae. Light-mediated water column transformations positioned below the sun. Photodegradation of  $\text{CH}_3\text{Hg}^+$  results in mostly  $\text{Hg}^0$  and an unknown  $\text{C}_1$  species depicted as  $\text{C}_x$ .

## **OBJECTIVES:**

The ecological distribution of MRB in different areas might be dependent on population and types of heterotrophic bacteria as well as on the degree of pollution due to mercury and other toxicants. A comparison of abundance of MRB with respect to time and space might be very useful in detecting coastal heavy metal pollution and such data on MRB can even be useful in efforts aimed at detecting coastal pollution. Resistance to mercury and/or other toxic materials might prove useful in biotechnological processes by facilitating any biological process, bio-mining of expensive/scarce metals or in the process of bio-remediation. Bio-remediation may involve the ability of the native microorganisms to remove any particular waste or use of microorganisms capable of degrading a variety of wastes including organic pollutants, mercury and/or other heavy metal contaminants. Keeping in view of the enormous amount of work on MRB worldwide as well as a major lack of studies from the Indian coasts, this research was planned to pursue the following major objectives.

1. One of the primary objectives of this study was to isolate and enumerate bacterial populations capable of tolerating mercury and to compare their occurrence and distribution along the Indian coasts. In addition, as there were data on MRB abundance collected variously during 1993-1997, it was also set out to evaluate whether temporal differences in their abundance were discernible.
2. One of the hypotheses this research work was based on was that bacterial strains capable of Hg resistance can also tolerate, detoxify or biotransform a variety of other toxicants. Thus, it was planned to delineate the potential of a select set of MRB to tolerate and/or biotransform polychlorinated biphenyls (PCBs), tri-butyl tin (TBT), cadmium and lead.

3. Upon selecting many MRB strains that were able to grow in the media containing various toxicants individually, further analyses were conducted to quantify detoxification/removal/precipitation of these toxicants with a view to understand the potential of these marine MRB for bioremediation.
4. It was also planned to understand the molecular mechanisms of *mer* genes in marine MRB. Most of the studies on mercury-resistance have been carried out from the freshwater environment. Marine, especially coastal, waters experience adverse effects of a variety of toxic chemicals. Moreover, characteristic salt concentration of marine environment may play a role in resistance mechanism in mercury-resistant marine bacteria. Whether altered concentration of NaCl retard/accelerate Hg removal from the growth media as well as dispersible effluents was also studied with a view to realize the salt requirement of certain marine MRB isolates.