

6.1. INTRODUCTION:

Biodegradation is nature's way of recycling wastes, or breaking down organic matter into nutrients that can be used and reused by other organisms. In the microbiological sense, "biodegradation" means that the decaying of all organic materials is carried out by a huge assortment of life forms comprising mainly bacteria and fungi, and other organisms. This pivotal, natural, biologically mediated process is the one that transforms hazardous toxic chemicals into non-toxic or less toxic substances. In a very broad sense, in nature, there is no waste because almost everything gets recycled. In addition, the secondary metabolites, intermediary molecules or any 'waste products' from one organism become the food/nutrient source(s) for others, providing nourishment and energy while they are further working-on/breaking down the so called waste organic matter. Some organic materials will break down much faster than others, but all will eventually decay.

By harnessing microbial communities, the natural "forces" of biodegradation, reduction of wastes and clean up of some types of environmental contaminants can be achieved. There are several reasons for which this process is better than chemical or physical processes. For example, this process directly degrades contaminants rather than merely transforming them from one form to the other, employ metabolic degradation pathways that can terminate with benign terminal products like CO₂ and water, derive energy directly from the contaminants themselves, and can be used *in situ* to minimize the disturbances usually associated with chemical treatment at the clean-up sites. Biological degradation of organic compounds may be considered an economical tool for remediating hazardous waste-contaminated environments. While some environments

may be too severely contaminated for initial *in situ* treatment to be effective, most contaminated media will use some form of biological degradation in the final treatment phase.

One of the common approaches to investigate bioremediation potential for recalcitrant pollutants such as PCBs, TBT or PAHs has been to isolate environmental strains using selective enrichment with more easily degraded analogues. For example, Chen & Aitken (1999) described the ability of *Pseudomonas saccharophila* to degrade the PAHs like benzo anthracene or pyrene based on the enrichment of a culture on phenanthrene. Microbiological means of biodegradation of xenobiotics like PCBs and TBT are important from the viewpoints of environmental safety concerns. This chapter provides results obtained from studying marine MRB potential for transforming PCBs and TBT besides documenting available literature.

Polychlorinated biphenyls (PCBs) were first synthesized in 1881 and, their first commercial production in 1929 in USA was an industrial breakthrough that prevented disasters such as electrical fires. Their mass production was started in late 1940s for a variety of industrial purposes, such as production of heat-resistant oils for transformers and capacitors, organic diluents, pesticide extenders, dust-reducing agents, printing inks, flame retardants, carbonless copy paper and many other products. PCBs were found to have low reactivity, high electrical resistance, and stability under heat and pressure, making them ideal for applications in rigor-demanding situations (Kimbara, 1999). But these same properties have allowed PCBs to accumulate and persist in the environment (Boyle et al., 1992). Recently in 2001 at the Stockholm convention, PCBs have been included in the list of persistent organic pollutants (POPs) and UNEP promulgated that all the PCB usage should be stopped until 2025.

Although not directly carcinogenic or neurotoxic, some PCBs are highly bioaccumulative due to their low water solubility ($\approx 10^{-5}$ to 10^{-11} moles) and low volatilization potential (Henry's Law (dimensionless) constants $\approx 4 \times 10^{-2}$ to 4×10^{-4}) (Schwarzenbach et al., 1993). Nonetheless, some PCBs are considered potential carcinogens because some of their mixtures have been shown to increase the development of hepatic tumors in rats (Safe, 1984; Kimbrough, 1987). Toxicity of different congeners of PCBs varies according to chlorine substitution at different positions of the biphenyl ring. It has been observed that DNA strand breaks occur in various species of marine organisms due to interaction with polychlorinated biphenyl contaminants having coplanarity (Dunn et al., 1987; Everaarts et al., 1994).

The physiological effects of PCBs vary from mammals, to birds, to humans. PCBs have been detected from wild animals since 1966, and 2 cases of mass poisoning caused by feeding rice oil contaminated with PCBs occurred on 1968 (Nagayama, 1976) and 1979 (Chen et al., 1980). PCB contamination has been observed in drinking water, sediments, wastewater, foods, and aquatic organisms (Boon et al., 1985, 1989). Estuarine and marine sediments are the ultimate global sinks for worldwide accumulations of PCBs sorbed to particulate materials (Kennedy, 1984) and environmental transformations of PCBs in the sediments have been documented (Berkaw et al., 1996).

Organotin compounds remained of purely scientific interest for a long time since they were synthesized in 1850. Their commercial production started in 1960's even though the first mention as a practical application of organotin compounds was made in a patent granted in 1943 that indicated their potential as antifouling compounds (Tisdale, 1943). The tributyltin compounds are a subgroup of the trialkyl organotin family of compounds. They are the main active ingredients in

biocides used to control a broad spectrum of organisms. Uses include wood treatment and preservation, antifouling of boats (in marine paints), antifungal action in textiles and industrial water systems, such as cooling tower and refrigeration systems, wood pulp and paper mill systems, and breweries. Non-point source of environmental exposure include discard and sanitary landfill disposal of plastic and direct release of biocides to freshwater or marine environment. TBT in antifouling paints is chemically bound in a copolymer resin system via an organotin-ester linkage but there is a slow and controlled release of this biocide, as the link gets hydrolyzed when seawater comes in contact with paint's surface (Evans, 1999).

Extensive use of organotins worldwide provoked scientific interest on the toxic effect of organotin compounds on aquatic and terrestrial biota (Smith, 1978, 1980, 1998). Realizing such possible environmental threats, the usage of TBT was banned way back in 1942 in France (Alzieu et al., 1986, 89). Most of the European countries, North America, Hong Kong however, imposed regulation on its usage in 1980s after it was in use widely for antifouling property since 1970 (Dowson et al., 1993; de Mora, 1996; Minchin et al., 1997; Evans, 1999, Champ, 2000, 2001). The International Maritime Organization (IMO) has already passed the resolution to ban on use of TBT-based antifouling compounds (Champ, 2000). Recent estimate shows that the annual world production of organotins may be close to 50,000 tons (Inoue et al., 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) and imposex in bivalves (Kiran & Anil, 1999; Barreiro et al., 2001). It has been reported that organotin compounds are toxic to both gram positive and gram negative bacteria but tri-organotins are more toxic to gram positive bacteria (Yamada et al., 1997). Inhibition of microbial

processes by TBT has been recorded for all major groups, with the main interactions occurring at cellular membranes and chloroplasts, or in the eukaryote mitochondria (Gadd, 2000).

For this study a mercury resistant marine bacterium CH07 was checked for its potential to degrade different congeners of PCBs from the technical mixture Clophen A-50 and, uniquely, was found to degrade PCBs belonging to different classes including many highly chlorinated congeners within just forty hours. CH07 and GP15 were used successfully in degradation of TBT to a considerable extent proving their efficiency in bioremediation of xenobiotics like PCBs and TBT.

6.2. MATERIALS AND METHODS:

6.2.1. Degradation of PCBs:

A marine bacterium designated as *Pseudomonas* CH07 was tested for its potential to degrade PCBs when present in a mixture with several of the congeners containing >4 chlorine atoms on the biphenyl ring. The technical mixture of PCBs, Clophen A-50 was obtained from Bayer, Germany and the PCBs standards were from Promochem, Germany.

6.2.1.1. Growth of CH07 in medium containing Clophen A-50:

The growth of the isolate was determined in SWNB. Fifty microlitres of a 24 hr old culture of *Pseudomonas* CH07 was inoculated into two 250 ml flasks containing SWNB (100 ml) with hexane solubilised Clophen A-50 added to them to a final concentration of 100 ppm. A control, SWNB medium without any PCBs was included. Flasks were incubated on a rotary shaker (200

rpm) at room temperature (ca. 28° + 2° C) for 120 hour. The absorbance (OD₆₆₀) of the culture was measured every 12 h. Log values of cell numbers were plotted to draw growth curves.

6.2.1.2. Culture media and experimental methods:

A defined seawater nutrient broth (SWNB) medium containing beef extract 3 g l⁻¹, peptic digest of animal tissue 5 g l⁻¹ prepared with 500 ml seawater and 500 ml distilled water (final pH was adjusted to 7 using 0.1 N NaOH). After autoclaving, the required amount of stock solution (10,000 ppm in n-hexane) of Clophen A-50 (Bayer, Lot no. 16572) was added into the medium to achieve a final concentration of 100 ppm. Immediately after adding the stock PCBs solution, the hexane part of it was evaporated out by gentle swirling of the flask in sterile condition and sterile glycerol was added to the medium in a 1:1 ratio of stock solution:glycerol. Forty microlitres of 24 h old broth culture of *Pseudomonas* CH07 was added in two replicates of 20 ml test medium (SWNB+ClophenA-50) and normal SWNB (without Clophen A-50). Controls in duplicate were also maintained without the addition of the organism at room temperature (ca. 28°+ 2° C). At various predecided intervals of time, the samples were taken out aseptically and extraction of PCBs was carried out prior to GC analysis.

6.2.1.2.1 .Extraction of PCBs:

The PCBs were analyzed following the method described by Boon et al (1985, 1989). The method was standardized in our laboratory using the PCBs standards obtained from Promochem, Germany. The purity of the solvents was checked by Gas chromatography for each of the bottles. The different adsorbents, alumina, silica, anhydrous Na₂SO₄ and the glass wool were purified by Soxhlet extraction with di-chloro-methane (HPLC grade). Different steps of the analytical methods are listed below.

- Aliquot of 1 ml sample was treated with 1 ml n-hexane (HPLC grade) thrice and thoroughly mixed by a vortex mixture for five minutes each time. The upper part of the solvent layer (solvent extract) was transferred to a sterilized glass tube.
- The solvent extract concentrated to 1 ml by evaporation with Snyder column evaporator on a water bath at 85° C and purified by alumina clean-up using micro-column technique.
- PCBs were separated from polar chlorinated compounds by eluting through a silica micro-column.
- PCBs fractions were concentrated to 1ml by evaporation with Snyder column on a water bath at 85° C.
- Analyzed the aliquot by GC-ECD with reference to standard PCBs (individual congeners).

6.2.1.2.2. Gas chromatographic analysis of PCBs:

The samples were analyzed by gas chromatography (Varian GC-3380) coupled with an electron capture detector and an autosampler 8200. A capillary column VA-5 (30 m x 0.25 mm) was employed with electron capture detector (ECD) for peak detection. Argon with 5% methane was the carrier gas. Injector temperature was 250° C. These conditions yielded peaks that were well defined and well separated. Analysis of PCBs was calibrated using different dilutions of standards for individual congeners of PCBs obtained from Promochem, Germany. The linearity of the calibration curve was determined with a range of dilution of the mixed-individual-standards.

6.2.1.3 Identification of breakdown pathway:

Isolate CH07 was streaked on M9 (modified M9; see chapter 5) agar plate adding biphenyl crystal on the lid as the sole source of carbon and sealed with parafilm. In parallel, the M9 agar

plate was supplemented with yeast extract as carbon source. After 24 hours of incubation in the dark, the colonies were sprayed with 2,3 dihydroxybiphenyl (2,3- OHBP) an intermediate in the biphenyl degradation pathway to see if there was any appearance of yellow metacleaveage product (2-hydroxy-6-oxo-6phenylhexa-2,4-dienoic acid) which would have appeared if there was production of 2,3 dihydroxibiphenyl dioxygenase by *bphC* gene.

6.2.2. TBT degradation:

CH07 and GP15 were used in this experiment. Stock solution of Tributyltin chloride (E-Merck, Germany) was prepared in double distilled dichloromethane. A known, TBT-negative strain, MSB8 was used as a negative control.

6.2.2.1. Monitoring bacterial growth in medium amended with TBT:

Preculture was made by growing bacteria (CH07 and GP15) in M3 medium supplemented with 10 ppm TBT (as Sn) on a rotary shaker (200 rpm) at 30° C for 24 hours. Dark brown flasks were used for the whole experiment to avoid the effect of light on TBT degradation. In a 500 ml sterile flask 300 ml of sterile M3 medium (Mahtani & Mavinkurve, 1979) was mixed with TBT stock solution (10,000 ppm) to obtain final concentration of 10 ppm TBT. From this TBT-mixed 300 ml medium, 50 ml each was transferred in to 5 sterile 250 ml flasks. Two each of these flasks were inoculated with CH07 and GP15 precultures individually. Into the last flask, both the cultures were inoculated. TBT-sensitive bacterium MSB8 was inoculated as a negative control. Everyday the growth was monitored by measuring OD₆₆₀ for 10 days.

6.2.2.2. Measurement of TBT degradation by MRB:

Preculture was made by growing bacteria (CH07 and GP15) in M3 medium supplemented with 5 ppm TBT on a rotary shaker at 200 rpm at 30° C for 24 hours. As done earlier, 300 ml of sterile M3 medium was amended with TBT stock solution to a final concentration of 5 ppm and, 50 ml portions were transferred to five sterile flasks. Two each of these flasks were inoculated with CH07 and GP15 precultures individually and the remaining with both of these cultures. Samples were collected from each flask on day 1, 7 and 14 for analysis of TBT and its breakdown products following the methods of Matthias et al. (1986). While the growth was monitored by measuring OD₆₆₀ everyday, samples from day 2 (48 h), and 13 (312 h) only were taken up for analysis of TBT and its degradation products.

6.2.2.2.1. Extraction of TBT:

500 µl of sample was extracted with double distilled dichloromethane (CH₂Cl₂; DCM) in presence of sodium borohydride (NaBH₄) after adding appropriate amounts of tripropyltin (TPrT) as internal standard. The extraction was done in three steps. At first, 5 ml DCM was added, the test tube was vortexed for 10 minutes, kept undisturbed for 15 minutes to cool down and, the lower organic phase was collected in a separate test tube by a Pasteur pipette. At the second step, 5 ml DCM was added and the tube was vortexed for 5 minutes followed by a cooling step of 15 minutes and, then the organic phase was collected. The last step involved addition of 2 ml of DCM, 2 minutes vortexing and 20 minutes cooling prior to collection of the organic phase. Adequate amount of sodium sulphate (Na₂SO₄) was added to the collected sample and the sample was filtered through Whatman (No.1) filter paper. The sample was concentrated to 500 µl with nitrogen gas, dissolved in double distilled hexane, concentrated again finally to around 500 µl and stored in the freezer till analysis.

6.2.2.2. Analysis of TBT and its degradation product:

Standards were prepared with tributyltin (TBT), dibutyltin (DBT), and TPrT. Ten microlitres of TBT (≈ 12.1 mg), 10 μ l of DBT (≈ 20.3 mg) and 10 μ l of TPrT (≈ 12.9 mg) were dissolved in 50 ml of double distilled methanol. Ten microlitres from each of these standards was made up to 1 ml with methanol. Ten microlitres from each of this stock was added to 1 ml methanol and a standards-mix was prepared by extraction with dichloromethane following the procedure as was done in case of sample preparation.

6.2.2.3. Evaluation of cometabolism of TBT in a dilute nutrient medium:

In a separate set of experiments, the growth of CH07 and GP15 was examined by providing a one-fourth strength SWNB and 10 ppm TBT to check if TBT was also metabolized by these bacterial strains in the presence of their usual growth medium. Growth was monitored daily by measuring OD₆₀₀ for over a period of 10 days to evaluate if bacteria can grow at rates as fast as they do in normal strength SWNB as a result of cometabolism of TBT in medium supplemented with organic nutrients, though at a nominal, quarter strength. CH08 served as a control.

6.3. RESULTS:

6.3.1. Degradation of PCBs:

6.3.1.1. Growth pattern of CH07 in the presence of Chlophen A-50:

It was evident that there was no appreciable effect of 40 different PCBs in Chlophen A-50 on the growth of *Pseudomonas* CH07 (Figure 6.3.1.1). The bacterium attained the exponential phase within 12 hours of growth in presence of PCBs akin to that in the control flask.

6.3.1.2. Analysis of PCBs:

It was observed that many congeners of PCBs in Clophen A-50 were degraded by *Pseudomonas* CH07. Among the different congeners of PCBs present in Clophen A-50, 14 chlorobiphenyls were found to be degraded in varying percentages (Table 6.3.1.1). Of these, one coplanar congener (CB-126) 3,3',4,4',5-pentachlorobiphenyl and one sterically hindered (CB-181) 2,2',3,4,4',5,5'-heptachlorobiphenyl were completely degraded (Table 6.3.1.2). Two asymmetric di-ortho chlorinated biphenyls (2,2',4,5,5'-pentachlorobiphenyl, and 2,3',4,4',6-pentachlorobiphenyl) were found to be degraded to 20.19% and 19.66% respectively. One of the congeners (2,3,4,5,6-pentachlorobiphenyl) with all the chlorines substituted in one of the aromatic rings being highly polar, was degraded to only 20.04% (Table 6.3.1.1). These results are part of the granted US patent no. 6544773 dated, April 8, 2003 (Sarkar et al., 2003) and this isolate CH07 was deposited as NRRL B-30604.

6.3.1.3. Identification of breakdown pathways:

The marine MRB strain CH07 did not grow on M9 plates, which had parafilm, stuck biphenyl crystals on the lid where as it grew normally on the M9 agar plates supplemented with yeast extract as carbon source. The colonies on the latter medium when sprayed with 2,3 dihydroxybiphenyl (2,3- OHBP) were negative for metacleaveage product (2-hydroxy-6-oxo-6phenylhexa-2,4-dienoic acid) indicating the non-functioning or total absence of 2,3 dihydroxybiphenyl dioxygenase by *bphC* gene.

6.3.2. Degradation of TBT:

6.3.2.1. Growth of bacteria and cometabolism of TBT:

The pseudomonad, CH07 grew to a slightly higher cell density (10.03×10^9 cells ml^{-1}) when compared to GP15 (9.89×10^9 cells ml^{-1}) in growth medium amended with 10 ppm TBT. Metabolism of TBT appears to be faster in the presence of organic enrichment as can be seen from the Figure 6.3.2.1. In both the growth conditions, death phase was evidenced after ca. 192 hrs. In control flasks with no added TBT, both the bacteria grew better (maximum growth was 10.1×10^9 cells ml^{-1}) than in the test conditions.

6.3.2.2. Degradation of TBT:

The pseudomonad CH07 degraded the TBT faster than GP15 (*Alcaligenes faecalis*). At the end of the experiment i.e. after 312 hrs, CH07 degraded nearly 54% of the initial TBT concentration (approximately $3564.4 \text{ ng ml}^{-1}$) *vis a vis* ca 34% by GP15. Appearance of DBT in the media also increased with time and at the end of 312 hrs (Figures 6.3.2.2.1 and 6.3.2.2.2), DBT was 320 and 83.2 ng ml^{-1} in case of CH07 and GP15 respectively (Figure 6.3.2.2.3). Appearance of DBT in varying amounts (Figure 6.3.2.2.3) implies that these marine MRB strains were able to degrade TBT quite effectively. With organic enrichment, the amounts of TBT degraded were similar by both strains but the degradation rate was faster.

6.4. DISCUSSION:

Biodegradation is the key process in bioremediation of toxic organic chemicals beside several other processes like bioadsorption, bioabsorption and bioaccumulation. Isolation and study of pure cultures capable of degrading contaminants can aid the identification of major factors

limiting the degradation of recalcitrant contaminants. There has been significant research effort in this area. Isolation of organisms capable of degrading contaminants and experimental studies in pure culture, have been the mainstay of bioremediation research for much of the last thirty years (Rogers & McClure, 2003).

As the cleavage of chlorine is not usually easy, bacteria in general, can't use chlorinated aromatic hydrocarbons as substrate in particular when the environment has some other nutrient sources. The discovery of biological degradation of PCBs by microorganisms made in 1973 (Ahmed & Focht, 1973) paved way for PCB bioremediation research. Some PCB congeners are found to be transformed by both anaerobic and aerobic bacteria (Bradley et al., 1996; Kimbara et al., 1999). The aerobic degradation of PCBs is generally limited to less - chlorinated congeners (five or fewer chlorines per biphenyl molecule) by an enzymatic mechanism involving deoxygenation of the aromatic ring (Kimbara et al., 1999). Degradation of mono- di- and tri-chlorinated biphenyls is relatively common (Bedard, 1986). The more chlorinated congeners are generally recalcitrant to aerobic degradation and only few strains are capable of degrading PCBs with more than 4 chlorines.

However, the marine strain of MRB, CH07, was capable of degrading PCBs of different configurations. This is the first conclusive demonstration of an aerobic microbial process involving a marine bacterium that degraded either their single or multiple congeners of PCBs contained in Clophen A-50. Of the different congeners of PCBs present in Clophen A-50, two congeners (a 5 chlorine CB-126; 7 chlorine CB-181) were completely degraded. Of the three most toxic coplanar PCBs (4 chlorine CB-77, 5 chlorine CB-126 and 6 chlorine CB-169), this organism degraded CB-126 completely within 40 hours. The other coplanar PCB (3, 3', 4, 4'-

Tetrachlorobiphenyl, CB-77) was also degraded substantially within the same duration. Heptachlorobiphenyl CB-181 (2,2', 3,4,4', 5,6) was also degraded completely within 40 hours. Some of the exceptional observations from this study are that despite the presence of 13 PCBs with 5 or more chlorine molecules in Chlophen A-50. This MRB strain, CH07, was not only able to grow in the presence of such a mixture but also degraded, as detailed in the results, 14 congeners of PCBs. Although several studies have been interested in identifying potent PCB degraders, as Furukawa et al. (1979) observed long ago, it is generally believed that biodegradation of PCBs decreases with increasing chlorine substitution. Inability of the organism to grow on biphenyl as the sole carbon source indicates that the organism was unable to use biphenyl as carbon and energy source and it also further confirmed the absence of the complete *bph* operon. There was no production of yellow color metacleaveage product 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid after spraying the colonies with 2,3 dihydroxybiphenyl (2,3-OHBP). Also, it was found that there was no *bphA* gene, the characteristic gene for aerobic breakdown of PCBs in CH07. Also, a close match of diterpenoid dioxygenase (*ditA*) was found in this isolate, which is an indication of the possibility that there is a different mechanism of degradation of PCBs occurring in this bacterium. The product of this gene might be a dioxygenase, which might be capable of breaking down many organic moieties.

Some strains like *Pseudomonas* LB400, *Alcaligenes eutrophus* H850, *Corynebacterium* MB1 and *Acinetobacter* P6 are exceptional PCB degraders. The specificity of the dioxygenases in these organisms differs greatly. Strains P6 and MB1 studied by Bedard et al. (1987) are particularly active against double *para* chlorinated PCBs. H850 and LB400 preferentially express a 3,4-dioxygenase, forming a *cis*-dihydrodiol form 2,5,2',6'- tetrachlorobiphenyl, which is degraded faster by H850 than PCBs with an unchlorinated ring. The chlorobenzoic acids are not further

degraded by most PCB-degrading bacteria. An exception is the degradation of 2,3'-dichlorobiphenyl by LB400. The position of the chlorine is also important. Ortho positioning of two chlorines on a single ring resulting into sterically hindered PCBs greatly inhibits degradation. A combination of anaerobic followed by aerobic biodegradation has been found to maximize the removal of chlorine (Abramowicz, 1990). The most critical stage of the two-step, anaerobic to aerobic process is getting the anaerobic bacteria to proliferate at a new site, cometabolizing the higher chlorinated PCBs (greater than 4 chlorines per biphenyl). Because only aerobes are capable of degrading lower chlorinated PCBs (4 or less chlorines per biphenyl) it is important that anaerobes grow well, transforming as many highly chlorinated PCBs as possible to lowly chlorinated PCBs. Several factors affect the growth of bacteria. Briefly, these are chemical structure, initial concentration of PCBs, solubility and types of PCBs in the wastes, as well as environmental conditions (temperature, pH, salinity, redox potential, available carbon, presence of other contaminants). Generally, for mixtures of PCBs with less than four chlorine atoms, sufficient degradation may be achieved using a one-stage procedure involving only aerobes. There is a danger, however, if highly chlorinated PCBs are left intact because they are generally more bioaccumulative than the lowly chlorinated ones (Unterman, 1996). Additionally, there are the highly toxic congeners referred to as "dioxin-like", containing chlorine at the two *para* positions (4 on the biphenyl ring) and at least two chlorines at the meta positions (3 or 5 position). The lack of ortho groups allows the atoms in these congeners to line up in a single plane (referred to as coplanar PCBs) that makes them especially toxic. Luckily it is the *meta* and *para* positions that are more susceptible to dechlorination (Bedard & Quensen, 1995). In this regard, future studies subjecting all 209 congeners of PCBs to marine strains such as CH07 and alike might yield deeper insights for effectively mitigating the environmental PCB contamination in the environments.

The PCBs have impacted our environment through accidents, spills in industrial facilities and mismanagement of storage or disposal areas. The Environmental Protection Agency (EPA) regulates disposal of PCBs through the Toxic Substance Control Act (TSCA). The TSCA requires that PCBs be disposed in accordance with the methods regulated by the EPA. Incineration is the standard method of destruction for PCBs and the only one approved to remove PCB from the soil and sediment (Blake, 1994). Regulation provides alternate methods that could demonstrate the destruction of PCB equivalent to incineration.

Bioremediation via biodegradation is an alternative highly attractive method for the disposal of PCBs due to the high costs of transportation, incineration and other procedures of remediation that currently exist. The technology of bioremediation has countless advantages. Among them we can mention that it treats low concentration of contaminants; prevents physical and chemical treatment; maintains alteration of the contaminated area to a minimum; eliminates long term responsibility; can be combined with other treatment technologies for highly complex mixed waste; destroys organic contaminants; does not generate secondary waste and is cost effective (Churchill et al., 1995; McGraph, 1995; Sturman et al., 1995). As incineration is costly in terms of transportation and energy and leaves a scarred landscape that must be either remediated or restored, alternative technologies must be developed. Though PCBs present a challenge to bioremediation, it is a hopeful alternative technology, as landscapes would be cleaned with much less disturbance and cost. Results of this study have been very useful for advocating the possibility of remediating the POPs through the application of marine MRB.

Organotin degradation involves sequential removal of organic group from tin, which generally results in toxicity reduction (Blair et al., 1982; Conney, 1988, 1995). Several studies have been carried out to identify the mechanism of biodegradation of TBT to determine if the de-alkylation is successive i.e. tri to di followed by formation of monobutyltin and lastly tin. Dibutyltin was the initial degradation product detected in Toronto harbour sediments (Maguire et al., 1986), whereas MBT was the principal initial product in the San Diego Bay (Barug, 1981). Reports on microbial degradation of TBT are very few (Gadd, 1993; Dubey & Roy, 2003). Barug (1981) reported a *P. aeruginosa* degrading only upto 2.5 mg l⁻¹. Another strain, *P. aeruginosa* USS25 could resist and degrade more than 650 mg l⁻¹ TBTC in laboratory conditions (Roy et al., 2004). Studies on marine bacteria-mediated degradation of TBT are even fewer (Barkay & Pritchard, 1988; Roy et al., 2004). In this study that examined two marine MRB, CH07 and GP15, it was evident that the former has a very high potential to sequentially biodegrade TBT. With this strain degrading 54% of the initial TBT concentration within a week, it is possible to suggest that the potential of such environmental strains needs to be more thoroughly established. This can be substantiated by the appearance and increase of DBT in the media with time. Though no attempt was made to check whether DBT was further degraded to monobutyltin (MBT) or elemental tin (Sn), it was clear from the decrease of TBT and, as a consequence, appearance of DBT, in varying amounts, that these marine MRB were able to degrade TBT quite effectively. With organic enrichment, amounts of TBT degraded were similar by both strains but the degradation rate was faster. Results from cometabolism experiments are useful to recognize that TBT is usually worked upon by the native microflora with the wherewithal to breakdown TBT and, will continue to attack this toxic moiety.

Although bacteria, algae and fungi, have demonstrated the degradation of organotins, it must be stressed that information is still very limited in relation to physiology of the process, relationship with tolerance, influence of anionic radicals and relative importance of microbial and abiotic degradation in natural habitats (Blunden & Chapman, 1982; Gaad, 1993, 2000). Bioaccumulation of TBT by bacteria, cyanobacteria and microalgae has been studied sparingly as a mechanism of bioremediation (Cooney & Wuertz, 1989). A *Pseudomonas* sp. accumulated TBT upto 2% of its cellular dry weight, apparently by purely physico-chemical processes, e.g. adsorption, with the bulk of the organotin being associated with the cell surfaces (Blair et al., 1982). In some cases, it is conceivable that exclusion of TBT from cell interiors may contribute to survival (Pettibone & Coney, 1988) and this appears to be the case for melanized cell types of *Aureobasidium pullulans* (Gadd et al., 1990).

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). As Pain et al. (1998) reported most of the TBT-resistant bacteria are also resistant to six heavy metals (Hg, Cd, Zn, Sn, Cu, Pb) suggesting that resistance to both 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. Further, as reported by Fukagawa et al. (1993) and Suzuki et al., (1994) genes conferring resistance to organotin may be present on the chromosomes. However, as with other inorganic and organic forms of toxic metals, many microorganisms may exhibit organotin resistance or tolerance, with several examples of organotin biodegradation being reported (Cooney & Wuertz, 1989; Cooney, 1995). Yamada et al. (1997) reported that TBT and triphenyltin (TPT) concentrations in squid livers

were higher in coastal water than in the offshore waters and the concentration of TBT higher in northern than in southern hemisphere.

Increased NaCl concentrations reduced the toxic effects of tributyltin, with possible effects being due to Na⁺ and Cl⁻ moieties, as well as possible osmotic responses of the organisms, which include changes in intracellular compatible solutes and membrane composition (Cooney et al., 1989). Marine isolates used in this study were able to grow in salinities ranging from 15 ppt to 35ppt. As the experiments with TBT were carried out at quite a high NaCl concentration, it is possible to suggest that these marine MRB strains are effective in dealing with TBT in truly marine and estuarine salt concentration levels.

In conclusion, most aerobic bacteria, for which the PCB-degradative competence has been accurately determined, seem to attack only a few types of congeners. The extent of degradation of different classes of congeners of PCBs by CH07 was unique and is a clear indication that this bacterium can be used effectively for PCBs detoxification. This strain is capable of degrading different congeners of PCBs with varying degree of polarity and stereochemical asymmetry. Most importantly, highly chlorinated congeners, CB-180 and CB-181 were degraded by it. The extent of degradation of different congeners of PCBs in presence of other chlorobiphenyls is a clear indication that this bacterium can be used effectively for their detoxification. However, absence of *bphC* product 2,3-dihydroxybiphenyl dioxygenase indicates to absence of complete *bph* operon and also possibly different pathways of PCBs degradation, which need detailed further research. This unique bacterium and several other MRB strains such as GP15 have been able to degrade TBT to a considerable extent. Moreover, being MRB these bacteria are resistant

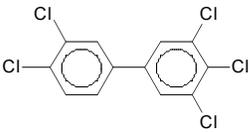
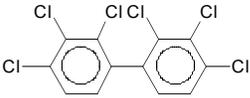
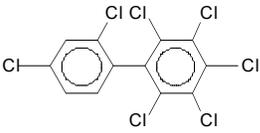
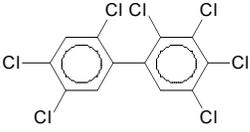
and useful in biotransformation of heavy metals like Hg, Cd or Pb as presented previously and, several other chemicals. They promise a great potential for environment clean up.

Table. 6.3.1.1. Degradation of different PCBs by a marine mercury resistant pseudomonad, CH07

Chlorobiphenyls	Molecular Formula	Conc. of PCBs in Control (ng/ml)	Conc. of PCBs in Test sol. (ng/ml) (incubation) 40 hrs.	Degradation of PCBs (%)
CB-101 (2,2',4,5,5' - Pentachloro)	C ₁₂ H ₅ Cl ₅	18.17	14.50	20.19
CB-119 (2,3',4,4',6-Pentachloro)	C ₁₂ H ₅ Cl ₅	8.07	6.48	19.66
CB-97 (2,2',3',4,5-Pentachloro)	C ₁₂ H ₅ Cl ₅	8.17	6.57	19.69
CB-116 (2,3,4,5,6-Pentachloro)	C ₁₂ H ₅ Cl ₅	10.09	8.06	20.04
CB-77 (3,3',4,4'-Tetrachloro)	C ₁₂ H ₆ Cl ₄	53.37	40.42	24.25
CB-151 (2,2',3,5,5',6-Hexachloro)	C ₁₂ H ₄ Cl ₆	2.04	1.28	37.32
CB-118 (2,3',4,4',5-Pentachloro)	C ₁₂ H ₅ Cl ₅	1.31	0.77	40.72
CB-105 (2,3,3',4,4'-Pentachloro')	C ₁₂ H ₅ Cl ₅	17.54	9.29	46.69
CB-141 (2,2',3,4,5,5'-Hexachloro)	C ₁₂ H ₄ Cl ₆	3.57	1.59	55.38
CB-138 (2,2',3,4,4',5'-Hexachloro)	C ₁₂ H ₄ Cl ₆	1.62	0.71	55.97
CB-126 (3,3',4,4',5-Pentachloro)	C ₁₂ H ₅ Cl ₅	2.75	00.00	100
CB-128 (2,2',3,3',4,4'-Hexachloro)	C ₁₂ H ₄ Cl ₆	5.02	1.79	64.33
CB-181 (2,2',3,4,4',5,6-Heptachloro)	C ₁₂ H ₃ Cl ₇	2.87	00.00	100
CB-180 (2,2',3,4,4',5,5'- Heptachloro)	C ₁₂ H ₃ Cl ₇	1.64	0.63	61.33

Table 6.3.1.2. Structures of PCBs degraded by a marine mercury resistant pseudomonad CH07

Chlorobiphenyls	Molecular Formula	Mol. Wt.	Cl (%)	Structures
CB-101 (2,2',4,5,5'- Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-119 (2,3',4,4',6-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-97 (2,2',3',4,5-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-116 (2,3,4,5,6-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-77 (3,3',4,4'-Tetrachloro)	$C_{12}H_6Cl_4$	220	64.54	
CB-151 (2,2',3,5,5',6-Hexachloro)	$C_{12}H_4Cl_6$	289	73.70	
CB-118 (2,3',4,4',5-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-105 (2,3,3',4,4'-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-141 (2,2',3,4,5,5'-Hexachloro)	$C_{12}H_4Cl_6$	289	73.70	
CB-138 (2,2',3,4,4',5'-Hexachloro)	$C_{12}H_4Cl_6$	289	73.70	

CB-126 (3,3',4,4',5-Pentachloro)	$C_{12}H_5Cl_5$	254.5	69.74	
CB-128 (2,2',3,3',4,4'-Hexachloro)	$C_{12}H_4Cl_6$	289	73.70	
CB-181 (2,2',3,4,4',5,6-Heptachloro)	$C_{12}H_3Cl_7$	323.5	76.81	
CB-180 (2,2',3,4,4',5,5'-Heptachloro)	$C_{12}H_3Cl_7$	323.5	76.81	

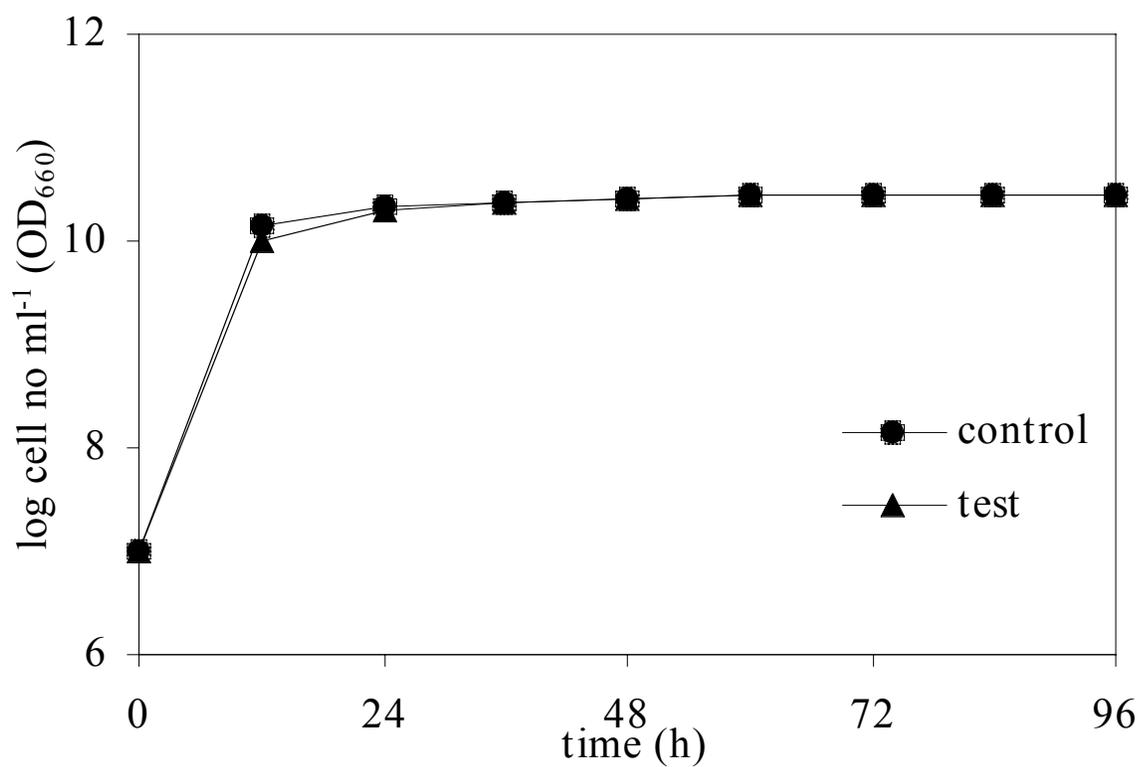


Figure 6.3.1.1. Growth of *Pseudomonas* CH07 in presence of 100 ppm Clophen A-50 in 50% seawater nutrient broth.

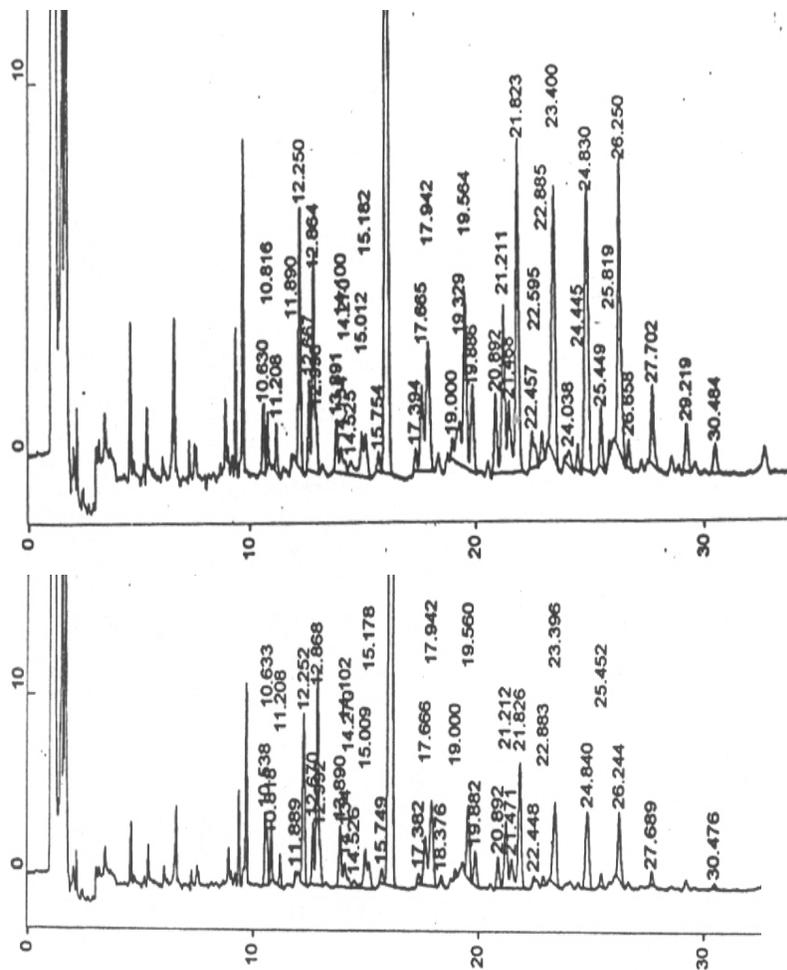


Figure 6.3.1.2. Gas chromatograms showing PCB degradation (upper panel represents "0" h and bottom one represent "40" h) by the marine mercury resistant pseudomonad, CH07

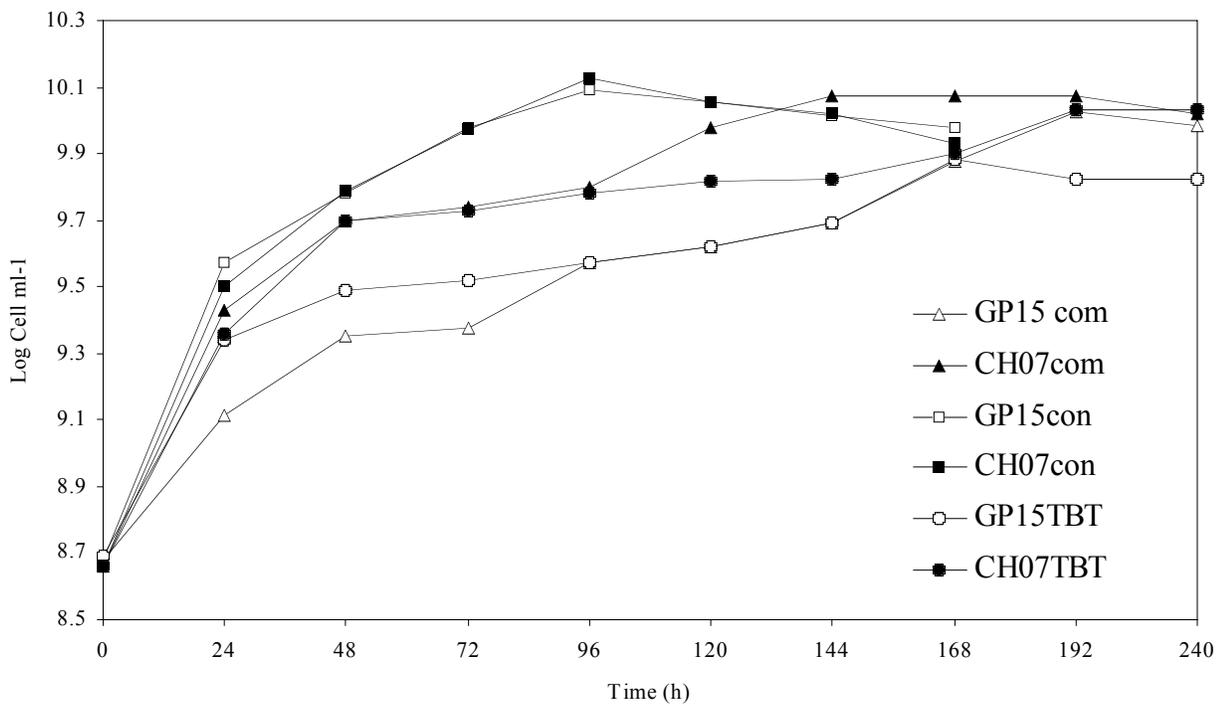


Figure 6.3.2.1. Growth of different marine mercury resistant bacteria (MRB) in TBT and effect of cometabolism. Conditions for these experiments are: con, control; com, cometabolism, TBT, medium containing 10 ppm TBT

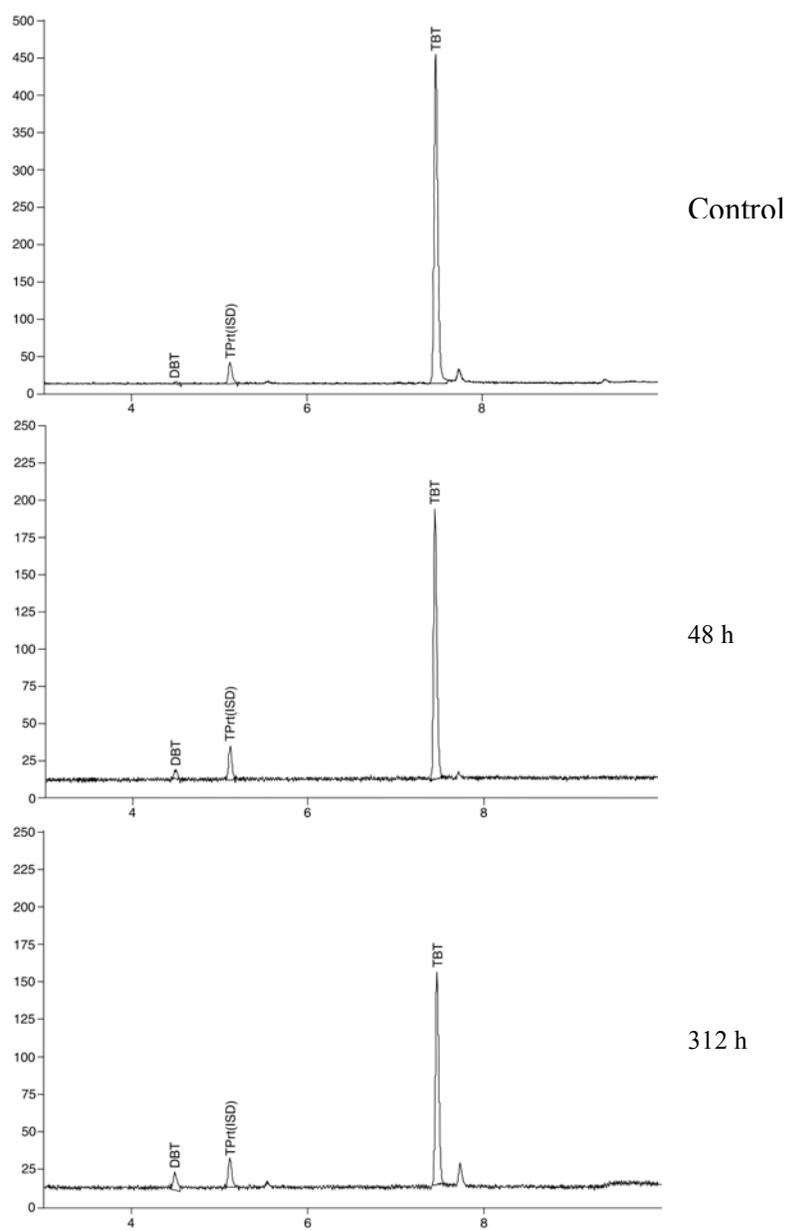


Figure 6.3.2.2.1. Gas chromatograms showing peaks of tributyltin, dibutyltin and tripropyltin for CH07.

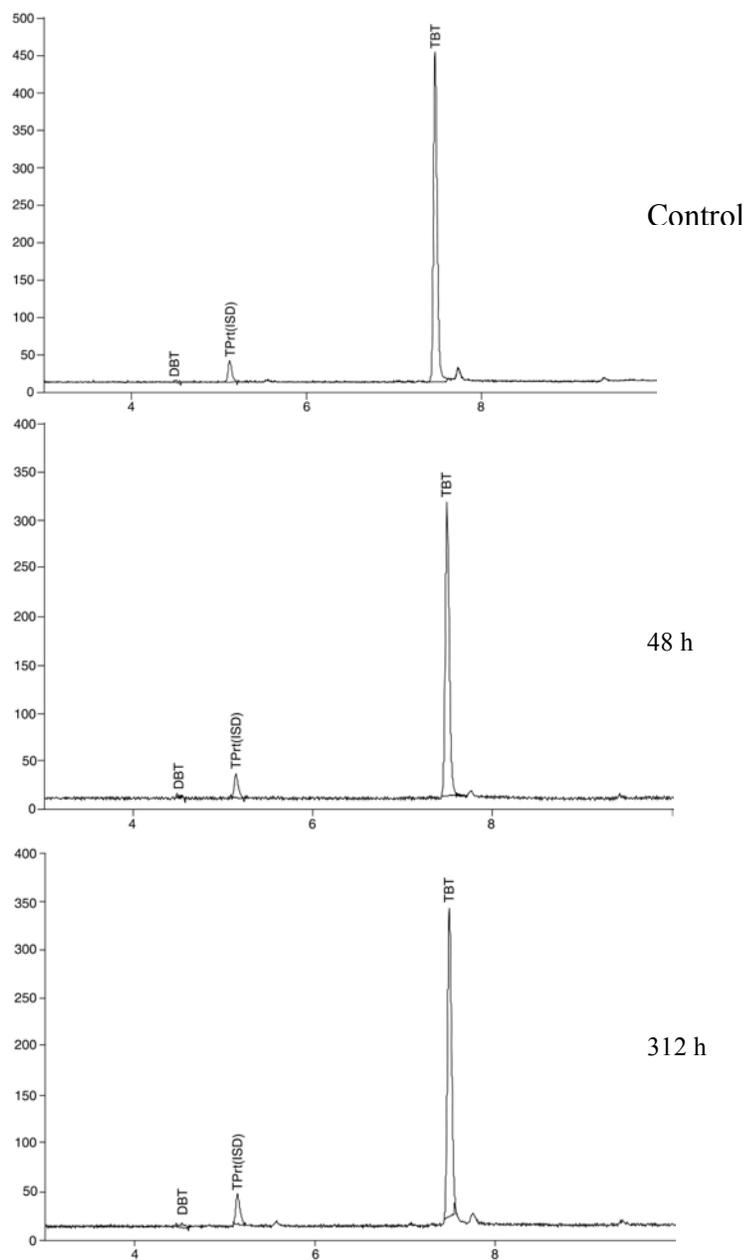


Figure 6.3.2.2.2 Gas chromatograms showing peaks of tributyltin, dibutyltin and tripropyltin for GP15

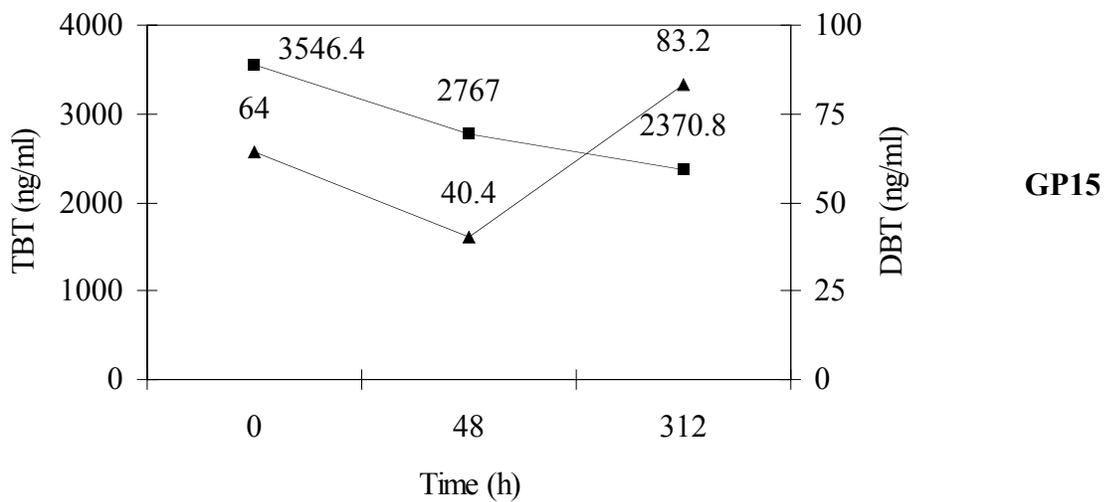
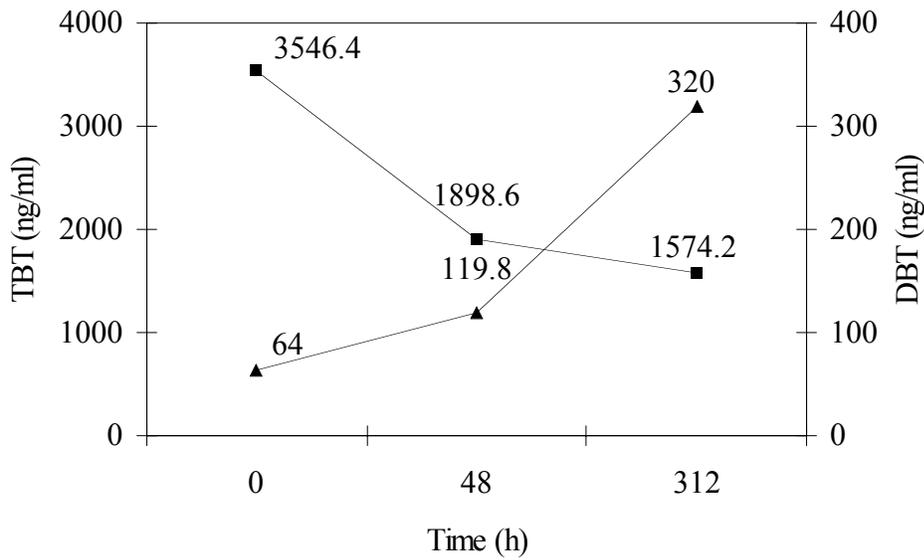


Figure 6.3.2.2.3. Degradation of tributyltin (TBT) to dibutyltin (DBT) by CH07 (*Pseudomonas aeruginosa*) and, GP15 (*Alcaligenes faecalis*). Line with filled square indicates TBT concentrations and the one with filled triangle, the DBT

7.1. INTRODUCTION:

Bioremediation is the term applied to technologies that accelerate natural processes for degrading and/or detoxifying harmful chemicals in soil, groundwater and wastewater. Bioremediation is defined as "the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals and other hazardous wastes" (Gibson & Sayler, 1992). This definition is accepted by the American Academy of Microbiology. Microbial bioremediation is the application/use of microorganisms to cleanup hazardous contaminants in soil, surface or subsurface waters, or wastewater. In the many forms of bioremediation, microorganisms are utilized and managed through the control of environmental factors to reduce environmental pollution. Modern biotechnology has selectively adapted naturally occurring microbes for their ability to detoxify specific toxic chemicals. When combined with nutrients, pH stabilizers, oxygen, and surfactants, these microbes attack the offending materials at a rapid rate to minimize contamination and reduce or eliminate the environmental hazard. Most microbial bioremediation processes take advantage of indigenous microorganisms. The objective of a microbial bioremediation program is to immobilize or to transform them to chemical products no longer hazardous to human health and the environment. For certain cases in which contaminants pose no significant risk to sensitive receptors (e.g., water supply wells, surface water bodies), intrinsic bioremediation may be an appropriate strategy. For other cases in which receptors are at risk, an enhanced (engineered) bioremediation strategy may be necessary. Enhanced bioremediation can be performed in-situ (e.g., biosparging; bioventing, hydrogen peroxide/inorganic nutrient amendment) or ex-situ (e.g., land farming, biopiles), depending on a variety of site-specific factors and the constraints imposed by site usage. In many instances, biostimulation activities may be limited to electron acceptor (e.g., molecular oxygen, nitrate, etc.) amendment. However,

in other cases, inorganic nutrient amendment or pH adjustment may be required. Typically, indigenous microbes are capable of effecting transformation because they are acclimated to the contaminant as well as their microniche. Research is underway at a number of facilities using exogenous, specialized microbes or genetically engineered microbes to optimize bioremediation (Dua et al., 2002). Intrinsic (passive) bioremediation of many synthetic organic compounds is carried out by indigenous microorganisms, principally heterotrophic bacteria that transform contaminants to intermediate products or innocuous end products. In many cases, contaminants such as petroleum hydrocarbons serve as sources of organic carbon and electron donors (assimilation). A successful, cost-effective microbial bioremediation program is dependent on hydrogeologic conditions, the contaminant, microbial ecology, and other spatial/temporal factors that vary widely. Microbiological assays are carried out to assess microbial growth conditions, degrader population densities and presence of enzymes capable of destroying contaminants of concern and microcosm studies to evaluate bioremediation potential under controlled conditions (Dua et al., 2002). During implementation of microbe bioremediation programs, performance monitoring plays a key role in evaluating treatment effectiveness. Properly executed, microbial bioremediation can cost-effectively and expeditiously destroy or immobilize contaminants in a manner that fosters regulatory compliance and is protective of human health and the environment (Roane & Kellog, 1996; Dua et al., 2002; Wagner-Döbler, 2003).

There are enormous variations in the levels of toxic mercury in the biosphere and no physiological roles for this metal have been documented, although a number of bacteria have adapted to this contaminant and have developed systems for metabolizing and utilizing the intrinsic energy of mercuric compounds to drive their own biosynthetic processes. Technologies for treating mercury-polluted environments have been a major concern over the last couple of

decades (Williams & Silver, 1984; Nakamura et al., 1990; Brunke et al., 1993; Canstein et al., 1999, 2000; Chang et al., 1997, Chen et al., 1998; Essa et al., 2002; Wagner-Döbler, 2003; Deckwer et al., 2004). Common methods to remove Hg^{2+} from contaminated water are mostly based on sorption to materials such as ion exchange resins (Osteen & Bibler, 1991; Ritter & Bibler, 1992). Removal of mercury in a laboratory test reactor using mercury-resistant bacteria was first reported in 1984 (Williams & Silver, 1984). Since then various attempts have been made to improve this technology. However, attention to bioremediation of this metal was seriously paid beginning 1990s (Summers, 1992). One of the initial efforts to retain mercury in bacterial bioreactors was made by Brunke et al. (1993). Reduction of Hg by MRB as the best such mechanism for its removal from chloralkali waste has been demonstrated (Canstein et al., 1999; Wagner-Döbler, 2003). Biosorption, another biological method involving adsorption of metals into the biomass such as algal or bacterial (either dead or alive), has been inexpensive and also promising (Chang & Hong, 1994; Mulligan, 2001), but is only applicable to low concentration of metals in water (Chen et al., 1998). Saouter et al. (1994, 1995) reported their preliminary investigation on using Hg^{2+} reducing strains to decontaminate a polluted pond in Tennessee. Diels et al. (1995) reported bioprecipitation of metal ions on the cell surface as a removal mechanism. Biosorption using natural (Volesky & Holan, 1995) or recombinant microbial biomass (Pazirandeh et al., 1995) has been also tried successfully. Chen & Wilson (1997a, 1997b) used genetically engineered *E. coli* expressing mercury transport system and metallothionein for removing Hg. Chang & Law (1998) developed a detoxification process using *P. aeruginosa* PU21 in batch, fed batch and continuous bioreactor system. Canstein et al. (1999) demonstrated the removal of mercury from chloralkali electrolysis wastewater by a mercury resistant *Pseudomonas putida* strain. 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). Recently, Deckwer et al. (2004) have described a three-phase fluidized bioreactor system. Alternative to bioreactor system using biofilm technique, available biological technologies include mainly sorption and precipitation as HgS. In spite of large initial interest using sorption as removal technology, no initiative has been taken for commercial application (Gadd, 2000). In this study, remediation of Hg by MRB was examined in a bioreactor, by monitoring the growth of a marine phytoplankton in MRB treated algal growth medium and by inoculating MRB into a saline soil to check if the MRB-treated soil helped the growth of a salt tolerant rice variety.

7.2. MATERIALS AND METHODS:

7.2.1. Remediation of Hg by MRB in a bioreactor:

7.2.1.1. Set up of the reactor:

The whole set up consisted of six reactors in parallel with each two of the reactor vessels containing the same bacterial inoculum. Each reactor unit consisted of main parts namely, reactor vessel filled with pumice granules (80 ml measured by water displacement), one side arm glass tube containing provisions for inlet and outlet and also a bubble trap and one glass tube for inoculating the reactor with the bacterial culture (100 ml total volume). The complete set up (plate 7.3.1) was sterilized by autoclaving and was placed inside a fume hood with provisions for disposal of waste effluent.

7.2.1.2. Functioning of the reactor:

The reactors were run in sterilized condition to measure the efficiency of the bacteria in removal of Hg either singly or in combination. They were all connected to separate medium reservoir and one waste supply via separate routes. Medium and wastewater (with different concentrations of mercury viz., 1, 2, 3.32, 4, 5.08, 6 and 8 ppm containing a varied range of salt concentrations ranging from 8 g l⁻¹ to 16 g l⁻¹) were pumped in an up-flow mode. The media and waste were always used after sterilization and the metal was added in sterile condition before subjecting them to bacterial action in the reactor. The mercury-containing waste and medium (modified M9 medium supplemented with 4 g l⁻¹ glucose) were pumped into the reactor under controlled speed. Mechanical disturbances like blockage of pipes, occurrence of bubbles inside the bioreactors causing disturbances to the functioning of the reactors were corrected from time to time. Mercury in the out-flow was measured using cold vapor atomic absorption spectrophotometry (CVAAS).

7.2.2. Remediation of Hg from phytoplankton growth medium by MRB

A marine cyanobacterium *Phormidium* sp. was grown as axenic population in ASN-III medium (one liter of this medium contained NaCl, 25 g; MgSO₄·7H₂O, 3.5 g; MgCl₂·6H₂O, 2 g; NaNO₃, 0.75 g; K₂HPO₄·3H₂O, 0.75 g; CaCl₂·2H₂O, 0.5 g; KCl, 0.5 g; Na₂CO₃, 0.02 g; Citric acid, 3 mg; ferric ammonium citrate, 3 mg; Mg EDTA, 0.5 mg; Vitamin B₁₂, 10 µg, A-5 trace mineral solution, 1 ml containing H₃BO₃, 2.86 g l⁻¹, MnCl₂·4H₂O, 1.81 g l⁻¹, ZnSO₄·7H₂O, 0.22 g l⁻¹, NaMoO₄·2H₂O, 0.39 g l⁻¹, CuSO₄·5H₂O, 0.079 g l⁻¹, Co(NO₃)₂·6H₂O, 49.4 mg l⁻¹ at pH 7.3±0.2; Rippka et al., 1981). Cultures were maintained at 29 ± 2⁰ C with photon flux density of 25-30 µ moles m⁻² sec⁻¹ in 12:12 h light: dark cycle. The minimal inhibitory concentration (MIC) of mercury (HgCl₂) for this cyanobacterium was determined by inoculating exponentially growing culture in ASN-III medium amended with various concentration of Hg ranging from 10 ppb to

200 ppb. Growth in terms of chlorophyll *a* was estimated by acetone extraction method (Kaushik & Goyal, 1993). Two MRB namely CH07 and S3 were used to detoxify ASN-III medium amended with 10 ppm mercury (HgCl₂). After 7 days, the culture was filtered through 0.2 µm membrane filter to exclude the bacterial cells. The filtrate after supplementing with mineral salts was inoculated with cyanobacteria in two different sets (one set with filtrate from treatment by CH07 alone and another with mixed cultures of CH07 and S3). Once the algal growth became visually apparent, chl *a* was measured after acetone extraction.

7.2.3. Bioremediation of Hg-contaminated soil for growing a salt tolerant rice

7.2.3.1. Preparation of soil:

The soil was taken from a rice-field adjacent to River Zuari. The farmer allowing inundation with estuarine water as and when desired manages this field. The soil was mixed thoroughly. One Kg of it was placed in a plastic tray to form ca 10 cm bed. Nine such trays were prepared for the experiment. The soil in these trays was seasoned in the laboratory with seawater (to a final concentration of 25% v/w of soil) for a fortnight before undertaking experiments with Hg.

7.2.3.2. Growth of MRB:

CH07 and GP15 were inoculated in SWNB containing 1 ppm Hg. Five hundred ml of fully grown culture (OD₆₆₀=1.2) was used for inoculating the soil.

7.2.3.3. Bioremediation of soil:

Known concentrations of Hg were added to different sets of experimental trays in the following manner. No Hg was added to three trays designated as controls (trays serially numbered 1, 2 and

9). Two trays (# 5 and 7) were spiked with 10 ml of 1000 ppm stock to arrive at ca 10 ppm (w/v) Hg in each tray. Another set of two trays (#6 and 8) were spiked with 15 ppm Hg. Soil in trays 5, 6, 7 and 8 were flooded with 500 ml broth cultures two days before the seeding was done. Soil in trays 5 and 6 were inoculated with CH07 whereas 7 and 8 were inoculated with a combination of CH07 and GP15. Soil in the tray 9 was inoculated with CH07 and this served as a control to see the effect of bacteria on the growth and survival of the khazan (salt tolerant) rice known commercially as Jyoti. The sprouting of rice-seedlings was achieved by holding the seeds in a moist condition. After two days, stock solution of Hg was added to soil in trays 3 and 4 to attain 10 and 20 ppm Hg (w/v) respectively in these trays. On the very next day, the sprouts of paddy were sown by properly positioning (sprout-up and root-down) in the trays keeping approximately 2 inches gap in between the two seedlings to thus have 9-12 rice-plants per tray. The trays were watered regularly and growth of the plants was measured in terms of height.

As there was no survival of even a single seedling in the trays with both these bacterial cultures, it was felt that they might be harmful to this rice. However, in order to check if the Hg remediation action by these bacteria continued, all the Hg spiked trays were replanted by the rice sprouts after a gap of 18 days. Day to day details of the experimental observations following the seasoning of the soil was noted for evaluating the Hg remediation by these two isolates of bacteria (Table 7.3.3.1).

7.3. RESULTS:

7.3.1. Remediation of Hg by MRB in a bioreactor:

Two *P. aeruginosa* (CH07 & Bro12) strains reduced mercury concentrations quite efficiently in the bioreactor for over a month's time (Figure 7.3.1). An amount of 192.65 mg was passed through each reactor totaling to 1.16 g during this operation and a total of 784.74 mg (67.88%) was recovered from the carrier bed (Table 7.3.1.1). The retention and recovery of Hg in the replicates of reactor-vessels was quite varying in the case of reactor vessels set up for CH07 unlike those for Bro12 where recovery of 56 and 60% Hg was recorded. The two reactors run with mixed cultures of CH07 and Bro12 resulted in recovery of 60 and 66% Hg (Table 7.3.1.2). Though mercury retention in the bioreactor by MRB were governed by many mechanical disturbances such as pipe blockage, bubbles in the reactor, faster flow rate, these two strains were able to retain 42 to > 95% (averaging ca 64%) of influent Hg (Figure 7.3.1).

7.3.2. Remediation of Hg from phytoplankton growth medium:

Phormidium sp. examined in this work could tolerate 100 ppb ($\mu\text{g l}^{-1}$) beyond which no growth was discernible. Thus, the MIC for this cyanobacterial species appeared to be ca 100 ppb (Table 7.3.2.1). When inoculated into medium containing 10 ppm Hg, there was no sign of viability in the culture. But when introduced into medium after growing CH07 and S3 in the ASN-III medium and then removing bacterial cells, the cyanobacteria grew quite well and rapidly as observed through chl-*a* increments (Table 7.3.2.2.). This was useful to suggesting a reliable bioremediation of Hg by MRB, which allowed good growth of *Phormidium* sp. in MRB-treated ASN-III medium.

7.3.3. Remediation of Hg from soil used for growing salt tolerant rice:

Primarily results of this study yielded three important observations. The salt tolerant rice grew in the soil amended with Hg at a concentration as high as 15 ppm (plate 7.3.3. -A). But the toxic effect of Hg resulted in wilting of the paddy leaves and the plants died within two weeks of sowing. Secondly, it was apparent that bacteria did not allow the sprouts to grow probably due to its pathogenicity to the rice (plate 7.3.3. -C). Most importantly when the soil was treated with MRB, all the seedlings not only survived (Table 7.3.3.2) but grew healthily without any wilting. Thus, MRB apparently had detoxified the soil and, in the bioremediated soil the saplings grew well (plate 7.3.3. -D) similar to the growth in the normal soil (plate 7.3.3. -B).

7.4. DISCUSSION:

There are numerous practical reasons for selectively separating heavy metal ions of all types from aqueous media. A few obvious examples are the remediation of hazardous or radioactive wastes, the remediation of contaminated groundwater, and recovery of precious and/or toxic metals from industrial processing solutions. A variety of well-known techniques are available to the chemists or engineers for these tasks, including solvent extraction, ion-exchange chromatography and precipitation. In modern applications of these techniques, recovery and re-use of the extractant materials is becoming more and more important. This is being driven by tougher environmental regulations, high initial costs of new, more effective, and more selective extractants, and the need to minimize volume of waste destined for permanent disposal.

Mercury pollution from human activities causes severe and localized alterations in the environmental levels of mercury. Recently, attempts are being made to use *mer*-mediated resistance in environmental remediation of mercury pollution. Mercury removal processes utilize mainly physical and chemical approaches that involve either trapping or collecting mercury from the contaminated sites or the chemical precipitation of mercuric compounds. Such processes are costly and very often leave behind hazardous by-products. Remediation technologies based on mercury volatilization have been explored (Fry et al., 1992; Saouter et al., 1994; Gadd, 2000), but have never proceeded beyond laboratory scale as collecting this toxic heavy metal is technically tedious and expensive (Wagner-Döbler, 2003). Several researchers for example, Frischmuth et al. (1991) and Brunke et al. (1993) demonstrated that the elemental mercury formed could also be retained in a packed bed bioreactor consisting of inert porous carrier material that was covered by a biofilm of mercury-resistant bacteria. Mercury accumulated in the carrier material was in the form of droplets of Hg (Canstein et al., 1999, 2001). The best performance in bioremediation of mercury in a bioreactor system has been demonstrated by a study using several *P.putida* strains (Canstein et al., 1999). Nearly 97% of the mercury was recovered from a waste containing 3-10 mg l⁻¹ Hg. In such bioreactors, outflow concentration of Hg was independent of inflow Hg concentration (Wagner-Döbler, 2003). In the bioreactors run with CH07 and Bro12 during this study, more than 60% influent Hg was quite rapidly accumulated in the bioreactors. Though a consortium of different bacterial strains is reported to be far more effective Canstein et al. (1999), combining CH07 and Bro12 for experiments in this study were not quite encouraging. This might imply that the compatibility of the consortium-candidates is of greater relevance. Several carrier materials like sira, pumice, activated carbon, wood chips, cellulose fibres and synthetic fibers have been used and they all work effectively excepting for some small differences due to difference in effluent mercury concentration, differences in the distribution and

thickness of the biofilm on the carrier, porosity of the packed bed and general flow characteristics in the bioreactor (Wagner-Döbler, 2003). Recently Essa et al. (2002) have reported three - including a new- mechanisms of mercury detoxification of wastewater in one organism, *Klebsiella pneumoniae* M426. This reports of enzyme-mediated reduction, aerobic precipitation of ionic Hg^{2+} as insoluble HgS , as a result of H_2S production and biomineralization of Hg^{2+} as insoluble mercury-sulfur complex other than HgS . Kiyono et al. (2003) successfully used bioaccumulation as a measure of bioremediation of Hg using *mer-ppk* fusion plasmid and were able to remove mercury from low concentrations (10-20 μM) but the efficiency was not so good at higher Hg concentrations i.e., 40-80 μM due to inactivation of viable cells inside the alginate beads. Chen & Wilson (1997b) constructed a genetically engineered *E. coli* strain to simultaneously express a Hg^{2+} transport as well as metallothionein improving the limitation of trans-membrane transport of mercury but this also was effective at a low concentration of mercury since its capacity for Hg^{2+} accumulation was limited by the number of metallothionein molecules present in the cells. Some of these systems have also been tested in bioreactors (Chang & Law, 1998). These strains potentially might be useful for removing mercury from very dilute solutions (Wagner-Döbler, 2003). The *mer* operon has been expressed in the radio-resistant *Deinococcus radiodurans*, which tolerated extremely high dosage of radiation and might be useful for cleaning up of sites contaminated with radioactive wastes (Brim et al., 2000, Daly, 2000). Phytoremediation expressing *merA* and *merB* genes in plants (especially rhizospheres) have been investigated for clean up of contaminated soils (Rugh et al., 1998; Bizily et al., 1999; de Souza, 1999) and very recently genetically engineered rice (Heaton et al., 2003) has also been tried for bioremediation of mercury. But the Hg^0 produced this way is released into the atmosphere directly (Wagner-Döbler, 2003). Some remediation treatments used SRB as source of H_2S production for precipitating metals as sulfides (White et al, 1999), in aerobic condition HgS

is methylated to the most toxic methylmercury and for this reason anaerobic treatment of mercury-contaminated matrices require extreme safety measures.

Inhibition of photosynthesis activity by heavy metals has been considered as one of the key metabolic event leading to inhibition of phototrophic growth in cyanobacteria (Rai et al., 1991). Impact of mercury on cyanobacteria has been the subject of numerous studies (Thomas & Montes, 1978; Murthy & Mohanty, 1993; Pant & Singh, 1995). Increasing mercury concentration affects the PSII which changes the photosynthetic performance of cyanobacteria reducing the phototrophic growth (Lu et al., 2000). Studies carried out on *Phormidium fragile* by Khalil (1997) also showed decrease in biomass and chlorophyll *a*, with increasing concentration of mercury. The growth of *Phormidium* sp. in the bioremediated synthetic media as observed in this study, proves the potential as well as efficiency of the MRB in removal of Hg. Bioremediation of the agricultural soil gives a new dimension in research of bioremediation of soil using MRB. That the rice variety examined during this study was able to grow initially suggests that the rice can tolerate quite a lot of Hg. Unfortunately, as can be plausibly viewed; all the saplings appeared to be withered due to increasing concentrations of Hg in the plant leaves and straw. While the sprouts that grew quite normally following the treatment of the soil for 18 days with MRB, this simple yet effective experiment has proven that MRB are very useful in bioremediating saline soils that would be useful in cultivation of salt tolerant rice and/or other vegetation.

Mercury bioremediation thus by using these marine bacteria as stated here can principally be applied to bioremediation of most kinds of mercury contamination. This environment-friendly and economic bioremediation technique offers a highly efficient way to remove mercury from polluted wastewater and also bioremediate contaminated soil, making such sites reusable.

Table 7.3.1.1. Rates of retention of Hg ($\mu\text{g hr}^{-1}$) in the reactors

Days of operation	Hg inflow		Mercury retention rate in the reactors* ($\mu\text{g/hr}$)						NaCl (gm l^{-1})
	Conc. (ppb)	Rate ($\mu\text{g/hr}$)	Reactor 1	Reactor 2	Reactor 3	Reactor 4	Reactor 5	Reactor 6	
1-7	1000	80	65.35	66.47	69.78	72.98	62.17	71.15	8
8-11	2000	160	148.48	132.72	149.68	151.28	152.72	148.56	8
12-16	4000	320	274.82	291.78	306.05	303.65	314.62	292	8
17-22	3320	265.6	235.06	238.53	203.63	186.8	231.01	225.2	8
23-26	4000	320	273.4	274.72	301.16	250.96	299.92	246.12	8
27-28	6000	480	459.68	456.48	465.68	450.08	458.96	454.72	8
29-30	8000	640	614.32	615.6	630.24	626.24	621.28	615.36	8
31-32	8000	640	552.8	604.24	582.48	606.4	607.52	611.44	4
33	4000	320	249.76	249.76	249.76	249.76	249.76	249.76	8
34-35	4000	320	272.24	298.64	305.44	279.68	306.88	303.72	16

*Reactors 1 and 2 were inoculated with CH07; 3 and 4 inoculated with Bro12; 5 and 6, with the mixture of CH07 and Bro12.

Table 7.3.1.2. Total Hg recovered from the reactors inoculated with a pseudomonad, CH07 (in reactors 1 and 2), Bro12 in reactors 3 and 4 and with both CH07 and Bro12 mixture in reactors 5 and 6

Reactor	Total Hg (mg) inflow	Total Hg (mg) recovered	% Recovered
1	192.65	81.54	42.32
2	209.62	201.12	95.94
3	211.03	119.72	56.73
4	201.72	122.5	60.73
5	214.95	129.62	60.31
6	196.02	132.24	66.44

Table 7.3.2.1. Minimum inhibitory concentration (MIC) of mercury for *Phormidium* sp.

Sample	Sampling day	<i>Chl a</i> ($\mu\text{g } 100 \text{ ml}^{-1}$)
Initial	0	30.17
Control	7	127.29
5 ppb	7	141.86
10 ppb	7	115.89
20 ppb	7	44.92
50 ppb	7	1.19
100 ppb	7	0.59
120 ppb	7	0.0
200 ppb	7	0.0

Table 7.3.2.2. Chlorophyll *a* concentration ($\mu\text{g } 100 \text{ ml}^{-1}$) in the flask cultures of *Phormidium* sp. after removing Hg through bioremediation using the Pseudomonad CH07 (S3) and combination of CH07 and *Bacillus pumilus* S3 (M3) on day 7.

Sample	<i>Chl a</i> ($\mu\text{g}/100 \text{ ml}$)
Inoculum	1.93
Control (ASN-III)	127.29
CH07 (S3)	58.81
CH07 & S3 (M3)	17.46

Table 7.3.3.1. Experimental details of soil bioremediation

Days	Steps carried out
1	Preculture of CH07 and GP15 preparation in SWNB
2	Preparation of inocula for addition to beds in the trays
3	Spiking of soils with Hg in trays 5, 6, 7, 8 (soils under biotreatment)
4	Overlaying spiked soil in trays 5 and 6 with CH07 broth culture; trays 7 and 8 with mixed broth culture and soil in tray 9 with CH07 broth culture. Sprouting of rice seed.
5	Spiking of soils with Hg in trays and 3, 4 (no bacterial treatment)
7	Transplanting of sprouts
8-14	Watering of the plants and measurement of length of plants at regular interval.
15-23	Watering plants and measurement of height of the plants.
23-24	Sprouting of new rice seeds for continuing experiments in trays treated with MRB
25	Transplanting into trays where bacterial cultures were added 18 days back
29	Measurement of plant height
34	Measurement of plant height and photographs were taken

Table 7.3.3.2. Growth and survival of a salt tolerant rice variety, Jyoti when grown in mercury spiked soil including conditions before and after bacterial bioremediation of Hg in the soil

Experimental trays	Condition	Growth on different days					no. of sprouts planted/survived
		5	7	18	22	27	
1 ^a	No Hg & no MRB	3 ^b	20	died	-	-	12/12
2 ^a	No Hg & no MRB	3	20	died	-	-	12/12
3	10 ppm Hg & no MRB	1.8	10	died	-	-	12/7
4	20 ppm Hg & no MRB	1.8	7.5	died	-	-	12/7
5	10 ppm Hg & CH07	NG ^c	NG	NG	4.5 ^d	15	9/7 (after second time planting)
6	15 ppm Hg & CH07	NG	NG	NG	3 ^d	12	9/7 (after second time planting)
7	10 ppm Hg & CH07+GP15	NG	NG	NG	3 ^d	10	9/7 (after second time planting)
8	15 ppm Hg & CH07+GP15	NG	NG	NG	3 ^d	9	9/7 (after second time planting)
9	No Hg & CH07	NG	NG	NG	NG	NG	12/0

^acontrol trays without any added Hg or any MRB cultures; ^bincrement (height) growth of sprouts on different days; ^cnew set of sprouts planted on day 18 following the addition of MRB. All the first set of sprouts had died by day 5 indicating the ill effect of MRB on these rice seedlings. Following the second planting on day 18 (thus allowing MRB to remediate mercury in the elapsed period), most rice plants not only survived, but grew quite well without suffering any wilting. In addition, the growth was quite rapid compared to the first 7 days when rice sprouts were planted immediately after MRB were added to these trays

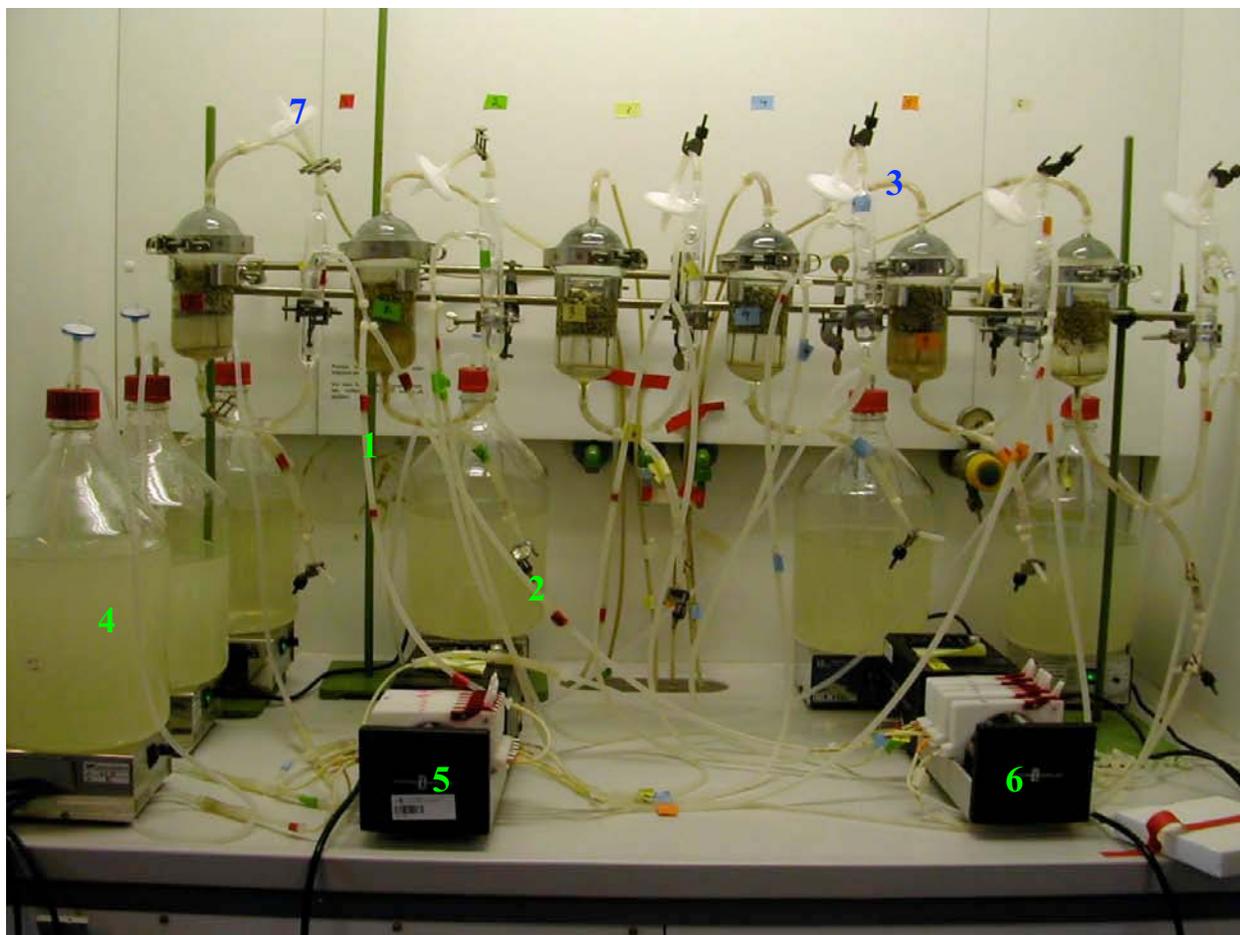


Plate 7.3.1. Bioreactor set up showing different reactor vessels and connections to influent and effluent Hg flows and other accessories. 1, channel for medium; 2, channel for wastewater inflow; 3, channel for wastewater outflow; 4, medium; 5, pump for medium; 6, pump for wastewater; 7, bubble trap

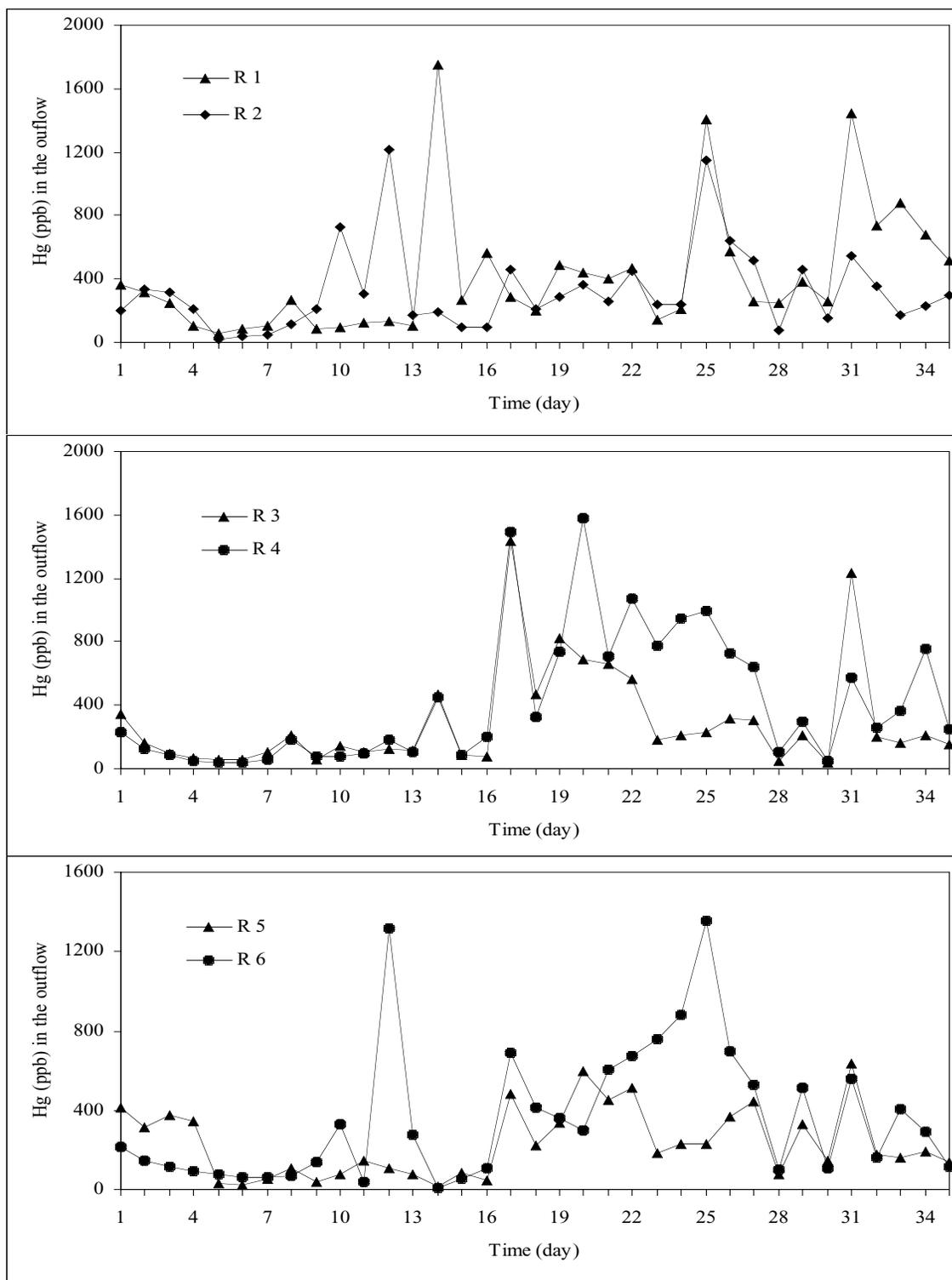


Figure 7.3.1. Mercury removal by CH07 and Bro12 in bioreactor
 R1 & R2, run with CH07; R3 & R4, run with Bro12; R5 & R6, run with mixed cultures



Plate 7.3.2. Growth of cyanobacteria in bioremediated Hg-complexed medium

Flasks labeled M3 denotes medium bioremediated with CH07; S3 denotes medium bioremediated with mixed culture of CH07 and S3

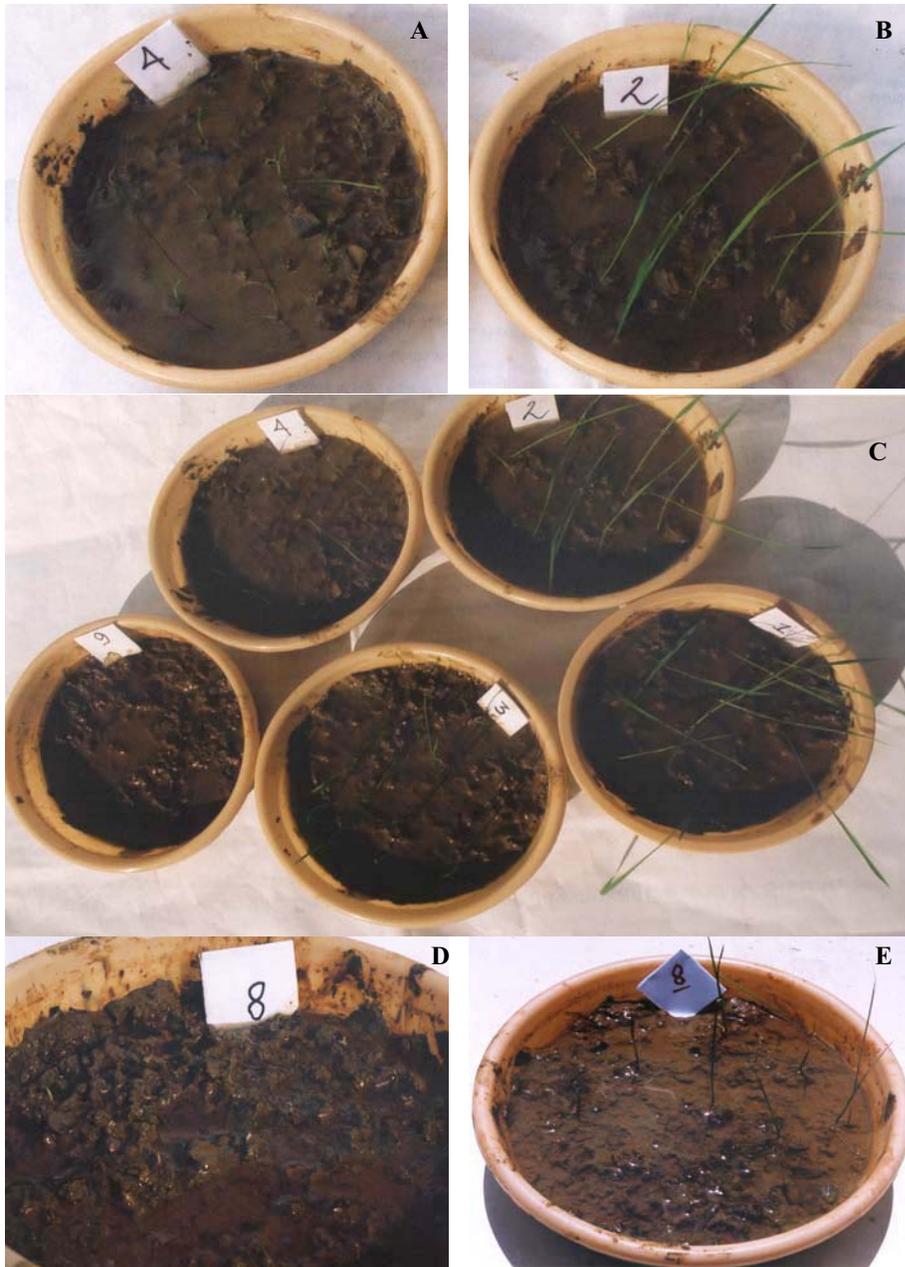


Plate 7.3.3. Bioremediation of Hg from saline (khazan) soil used for cultivating a salt tolerant rice variety, Jyoti. A, wilting of paddy leaves due to toxic affect of Hg [photographed on day 7 following placing sprouts]; B, normal growth of paddy [photographed on day 7 following placing sprouts]; C, normal, wilted and adversely affected paddy [photographed on day 7 following placing sprouts]; D, photograph showing the adverse effect of bacteria on growth of paddy [photographed on day 7 following placing sprouts]; E, no wilting of paddy leaves grown in bioremediated soil [photographed on day 9 following placing second set of sprouts]. See Table 7.3.3.2 for more details.