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Toxicity Assays and Bioconcentration of Mercury in Bacteria Selected from Marine Environments

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Heavy metals released in marine environments are of increasing concern. Owing to the importance of the marine microbial communities, there have been numerous studies to predict and analyze the toxic effect of heavy metals on natural communities (Suedel et al.1994). Biological parameters such as growth rate, biomass measurement and specific enzymatic activities are currently used to evaluate toxic effects on bacterial populations (Montuelle et al. 1994).

Although inorganic mercury (Hg) and organomercurials are among the most toxic metal compounds, several bacterial genera isolated from polluted environments in temperate regions exhibit some degree of resistance to Hg (Stein et al.1996). Some of the mechanisms involved in such process have been elucidated (Barkay. 1987; Fabiano et al. 1994; Ghosh et al. 1997). However, little is known about Hg pollution and microbial resistance in tropical marine environments.

The aim of the present study was to evaluate the toxicity of mercury to marine bacteria isolated from three different communities (coral reef, mangroves and *Thalassia testudinum*) present at the Morrocoy National Park, Venezuela. Toxicity assays were performed with increasing $HgCl_2$ concentrations. Tolerant strains were isolated to analyze the uptake kinetics of Hg.

MATERIALS AND METHODS.

Sediments (0.1 cm. of depth) and water samples were collected from three different communities (*Thalassia,* mangrove and coral reef) of Morrocoy National Park, on the west coast of Venezuela. This national park is in close proximity to the Golfo Triste industrial park, where a paper mill, oil refinery and petrochemical complex are among the most important industrial activities of the region.

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Water samples from which bacteria were isolated, were analyzed according to Standard Methods (APHA.1992) to determine salinity, temperature and pH. For the Hg analysis, samples of water and sediment were treated according to the protocol of the Environmental Protection Agency (1976) and analyzed after acid digestion by cold vapor atomic absorption spectrophotometry, using a Perkin Elmer Analyzer model 2380 (limit detection of 0.02 µg/ml). All samples were analyzed in duplicate.

Microbial cultures were grown in marine medium (MM) according to Gerald et al (1994) composed of nutrient broth 0.8%, casitone 1%, peptone 1%, dextrose 0.5%, tryptose 1% and sea water (pH 7.0). Water and sediments samples were inoculated in MM supplemented with $HgCl_2$ to reach a final Hg concentrations of 1, 10, 50 and 100 ppm (mg\L). The bacterial suspensions were incubated for 48 h. at 27°C and thereafter spread plated on MM agar. The isolates were obtained by selecting colonies from the higher concentration of Hg. Biochemical tests and Gram staining were performed for bacterial identification according to Bergey's (1994).

The toxicity tests of the isolated strains were performed in marine medium supplemented with $HgCl_2$ (1, 3, 5 and 7 ppm). Cultures both control and experimental were incubated for 24h at 27°C shaking at 140 rpm. Growth was monitored turbidimetrically at 600nm (Gerhard et al. 1994) with a Shimadzu 1200 UV Spectrophotometer. Viable counts were performed to determine colony forming units (CFU). The median inhibitory concentration (IC_{50}) was calculated by comparing the growth of the control with the results of CFU.

The same culture was simultaneously used to determine the intracellular concentration of Hg. Cells were harvested by centrifugation at 6000 x g for 10 min. at 0°C to 4°C. The pellet was resuspended in 5 mM EDTA to wash out the adsorbed Hg. (Shuttleworth and Unz. 1993). Cells were centrifuged again and both, the pellet and supernatants were recollected. Mercury concentrations of the fractions were determined in atomic absorption spectrophotometry. All Hg uptake experiments were done at least three times for each Hg concentration; every Hg concentration was determined in duplicate. The data were analyzed by the variance analysis (ANOVA). Significance levels were set at (P=0.05).

RESULTS AND DISCUSSION

The results obtained for the physicochemical parameters of the three environments were very similar to each other between the sampling stations: pH: 7.5, temperature: around 30°C., salinity: 38% - 39%, Hg concentration in water: from < 0.002 to 0.007µg/ml., Hg concentration in sediments: from <0.002 to 0.033 µg/ml.

Growth of bacterial population in $HgCl_2$ supplemented medium was determined visually as positive or negative. The results of this screening for all three stations are summarized in Table 1. From the results (Table1), it is evident that: (1)

Bacteria associated with the sediments are, in all cases, more tolerant to the $HgC1_2$ than bacteria from the water column. Abundance and bioavailability of Hg in the marine environment are proportional to the sediment-bound metal. Benthic organisms, chronically exposed to heavy metals associated to the sediment are prone to develop biochemical or physiological mechanisms to tolerate such conditions. It is not surprising that benthic organisms are more tolerant to mercury than plankton species (Bryian and Langston.1992). Direct evidence of the importance of particulates in the transfer of biologically available Hg is suggested from the data of Mikac et al. (1989) who found that the concentration of methyl mercury was higher in benthic species than in pelagic fish, feeding on low MeHg-containing plankton. The same pattern of resistance to mercury was found with bacteria from water and sediments (Nelson and Colwell.1975). (2) Microbial communities associated with the coral reef (water and sediments) showed low tolerance to Hg when compared to those associated to mangroves and *Thalassia*. Probably the calcareous coral sediment limited the Hg available to the bacterial community associated to the coral reef in the natural environment. Heavy metals tend to bind strongly to calcareous materials and the microbiota in such an environment is hardly exposed to its toxic levels of the metals.

It is unlikely to observe some tolerance to Hg in bacterial communities that have never been exposed to its toxic level of this metal. In contrast, in environments with high organic matter content, the heavy metal present in the sediments is more available for microorganisms. This availability becomes, over long periods, a pressure to select preferentially microbial strains able to growth in presence of heavy metals.

When mixed cultures of bacteria from each sampling station were selected in presence of high Hg concentration, two strains outperformed the rest of the bacterial community by their ability to grow in these conditions. Results of their morphological and physiological characterization are shown in Table 2. From the *Thalassia* station, a rod shaped bacteria belonging to the Vibrionacea family was isolated and was named Strain A. Strain B was isolated from the mangrove station and was identified as a species of *Staphylococcus*. Further characterizations of these strains are currently under way to confirm this preliminary identification.

Fig. 1 shows the effect of Hg on the growth rate of isolates A and B, as well as their tolerance to the toxic in short-term toxicity assays. The results can be summarized as follow: (1) Both isolates showed a similar growth pattern in the presence of Hg. (2) Increasing $HgCl_2$ concentration delayed the growth of both isolates, with a longer lag phase when concentrations of $HgCl_2$ were higher than 3 ppm. (3) At 7 ppm Strain B, but not A, grew.

The median inhibitory concentration (IC_{50}) at 12h was similar for both isolates (3 ppm). In contrast, at 24h, the IC_{50} for Strain A (4 ppm) was about half the inhibitory concentration found for Strain B (IC_{50} >7 ppm). This result suggested that the growth rate of Strain B was less sensitive to the toxic effect of $HgCl^2$ than Strain A. These growth differences were statistically significant (α =0.05).

Table 1. Primary pattern of growth in mixed cultures with Hg.

Station	Growth	Station	Growth
CRW **Control**	+	CRS **Control**	++
CRW 1 ppm	+	CRS 1 ppm	++
CRW 10 ppm	-	CRS 10 ppm	++
CRW 50 ppm	-	CRS 50 ppm	+
CRW 100ppm	-	CRS 100ppm	-
TtW **Control**	+	TtS **Control**	++
TtW 1 ppm	+	TtS 1 ppm	++
TtW 10 ppm	+	TtS 10 ppm	++
TtW 50 ppm	-	TtS 50 ppm	++
TtW 100 ppm	-	*Tt*S 100 ppm	++
MW **Control**	+	MS **Control**	++
MW 1 ppm	+	MS 1 ppm	++
MW 10 ppm	+	MS 10 ppm	++
MW 50 ppm	-	MS 50 ppm	++
MW 100 ppm	-	MS 100 ppm	++

++: Abundant growth
+:Normal growth
—: No growth
Control: without Hg.

CRW: coral reef water
TtW: *Thalassia* water
MW:mangroves water

CRS: coral reef sediments
TtS:*Thalassia* sediments
MS: mangroves sediments

Table 2. Morphological and biochemical characteristics of selected mercury-tolerant bacterial isolates.

Character	**Strain A**	**Strain B**
Sample site	*Thalassia*	Mangrove
Morphology	Rod	Coccus, in pairs
Color	Translucent white	Cream
Gram reaction	-	+
Motility	+	-
Respiration	Facultative anaerobic	Facultative anaerobic
Catalase	+	+
Oxidase	+	+ (weak)
Indole	-	-
MR	-	-
VP	+	+
Nitrate reduction	+	+
Citrate	+	-
Urease	-	+
Glucose	+	+
Lactose	+	+
NaCl tolerance	> 36%	> 36%
Tentatively Clas.	Family Vibronaceae	*Staphylococcus*

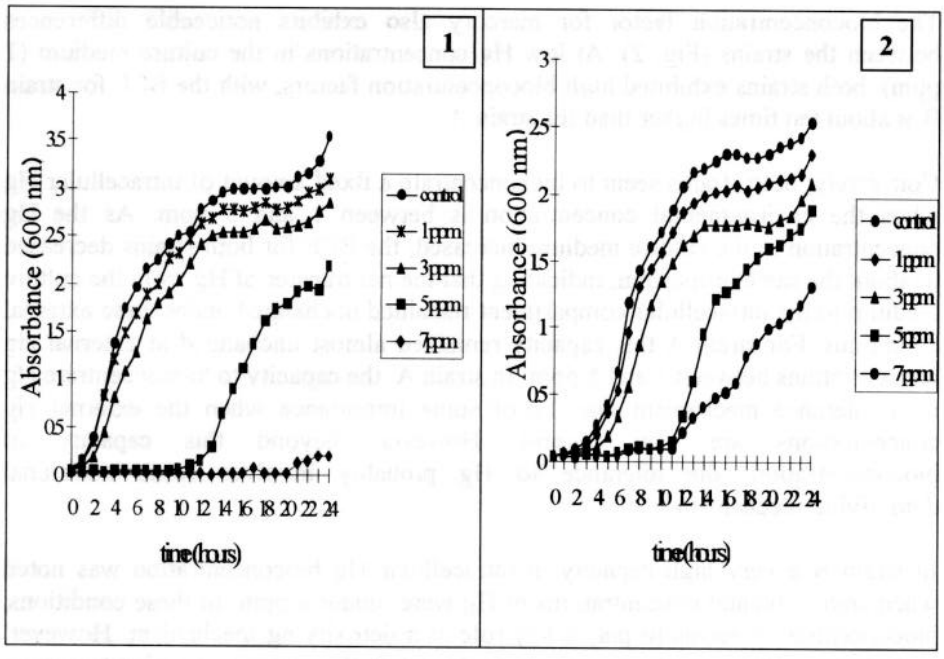

Figure.1 Effect of Hg on growth of **1:** Strain A (Gram negative rod). 2: Strain B (Gram positive coccus). Concentration (Hg ppm)

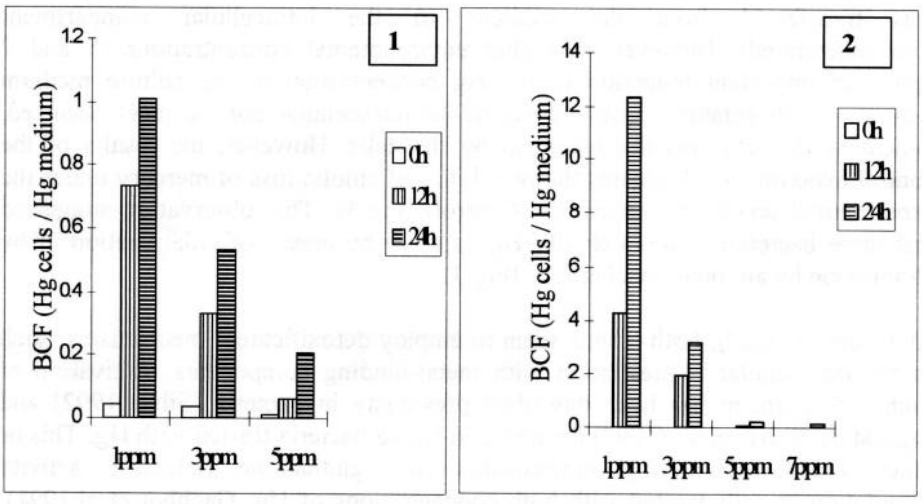

Figure 2. Bioconcentration Factor of Hg for different concentrations of Hg in the culture medium. (1) Gram negative rod. (Strain A). (2) Gram positive coccus. (Strain B)

The bioconcentration factor for mercury also exhibits noticeable differences between the strains (Fig. 2). At low Hg concentrations in the culture medium (1 ppm), both strains exhibited high bioconcentration factors, with the BCF for strain B is about ten times higher than for strain A.

Conversely, both strains seem to bioconcentrate a fixed amount of intracellular Hg when the environmental concentration is between 1 and 3 ppm. As the Hg concentration in the culture medium increased, the BCF for both strains decreased in about the same proportion, indicating that the net transfer of Hg from the culture medium to the intracellular compartment remained unchanged under these external conditions. For strain A this capacity remained almost unchanged at external Hg concentrations between 3 and 5 ppm. In strain A the capacity to bioconcentrate Hg as a tolerance mechanism may be of some importance when the external Hg concentrations are low (1ppm). However, beyond this capacity of bioconcentration, the tolerance to Hg probably involves some additional detoxifying mechanism.

In strain B a very high capacity in intracellular Hg bioconcentration was noted when environmental concentrations of Hg were under 3 ppm. In these conditions, bioconcentration seems to play a key role as a detoxifying mechanism. However, at higher environmental concentrations (5 ppm) the capacity to bioconcentrate appeared to be shut, and some alternate detoxifying mechanism may be responsible for the observed tolerance

Our results demonstrated that, at concentrations from 1 to 3 ppm of $HgCl_2$, the Hg was transferred from the medium to the intracellular compartment (bioconcentrated). However, at higher environmental concentrations (5 and 7 ppm), an important reduction in the Hg concentration in the culture medium without any detectable transfer of Hg to the intracellular compartment occurred, indicating that Hg was not taken up by the cells. However, the results of the control experiments eliminates the possibility of abiotic loss of mercury under the experimental conditions described (Controls, Fig.3). This observation suggested that these bacterial strains detoxify Hg partially by means of volatilization or by elimination by an other mechanism (Fig.3).

At 1 ppm of $HgCl_2$, both strains seem to employ detoxificateur mechanisms, such as the intracellular sequestration with metal-binding components. Activation of such a mechanism has been described previously in bacteria (Silver.1992) and suggest by Ghosh et al. (1997) for nitrogen-fixing bacteria treated with Hg. This or other similar detoxifying mechanisms (ie., gluthatione reductase activity stimulation in cells treated with high concentrations of Hg; Gachhui et al.1991), may be responsible for the intracellular Hg concentration that occurred at a Hg concentration around 1 ppm.

When Hg concentration was 3 ppm., three effects were evident: **(1)** Growth rate was severely affected, strain A being the most sensitive. **(2)** Inhibition of

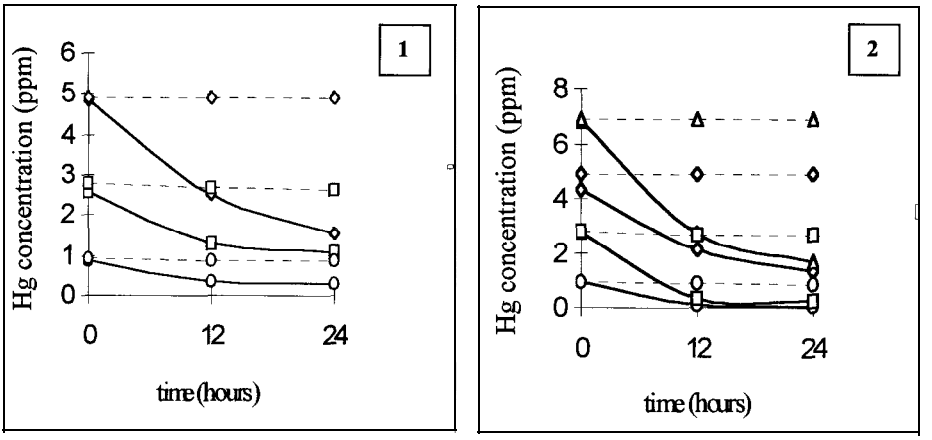

Figure 3. Culture medium Hg. concentrations control. 1. Strain A. 2. Strain B. o1 ppm. □3 ppm. ◊5 ppm. △ 7 ppm. (----) System Controls. (—)Treatments

the bioconcentration capacity. **(3)** Volatilization of Hg from the culture medium. These observations suggested that activation of mechanisms accounted for a significant fraction of the volatilized Hg. In some bacteria resistant to Hg, mercuric reductase volatilization is the most important detoxifying mechanism (Robinson and Tuovinen.1984). Activation of this type of mechanism is triggered only in presence of high Hg concentration (Gosh et al.1997); in our experiments, volatilization mechanisms may be active at Hg concentration around 3 ppm for both Strains, A and B.

However, mechanisms like cell envelope complexation or H_2S production have been identified in other different bacteria strains to be responsible of Hg elimination (Baldi et al. 1989; Stein et al. 1996). On the other hand, the availability of Hg for microbial transformations is lower in marine environments than in the freshwater systems, suggesting that other kind of detoxification mechanisms might be stimulated in the marine biota (Barkay et al. 1997).

The results presented here demonstrated that, in laboratory conditions, tropical marine bacteria tolerate mercury by the activation of several detoxifying mechanisms in combinations and proportions which vary according to the microbial strain and to the degree of Hg contamination. We also showed that pre-exposure remains a key factor in the capacity to tolerate Hg. It remain to be proved that the tolerance performance we observed under laboratory conditions remains the same when bacterial communities in their natural environment are exposed to Hg contamination.

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