

Bacteria as Potential Indicators of Heavy Metal Contamination in a Tropical Mangrove and the Implications on Environmental and Human Health

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ABSTRACT

Heavy metal (HM) exposure has been associated with human health diseases like cancer, kidney and liver damage, neurological disorders, motor skills, low bone density and learning problems. With the beginning of the industrialization, the heavy metals in high concentration contribute to putting on the risk the humans in the vicinity. Our study site is located in Cataño, Puerto Rico. This is a highly industrialized area. It is surrounded by a recreational park, a rum distillery, two thermoelectric factories, and was impacted by CAPECO (oil refinery) explosion in 2009. Las Cucharillas marsh is part of The San Juan Bay Estuary System, considered as a critical wildlife area. The mangrove marsh has three of the four mangrove species found in PR *Laguncularia racemosa*, *Avicennia germinans* and *Rhizophora mangle*. This study was aimed at seven different heavy metals: Arsenic (As), Cadmium (Cd), Chromium (Cr), Lead (Pb), Zinc (Zn), Mercury (Hg) and Copper (Cu). These metals at high concentrations are of human health concern due to their toxicity, persistence, bioaccumulative and bio magnification potentials. Contamination of surface sediments with HM affects the food chain, starting with marine organisms up to humans. The people who live near the contaminated area and the local fishermen are at high risk of exposure. Studies reveal that certain microorganisms can resist the toxicity of heavy metals even at high concentrations. Our study pretends to exploit the sensitive nature of some bacteria to HM and use them as bioindicators. The objective of this research is to assess the bacterial community on the mangrove marsh, identify these bacteria and correlate bacterial species with the type and concentration of the metals found on the site. Our preliminary results with the BIOLOG® identification were five bacteria that are: *Carnobacterium inhibens*, *Cupriavidus gilardii*, *Enterococcus maloduratus*, *Microbacterium flavescens* and *Ralstonia pickettii*. This study will continue with an assessment of the exposure of different concentrations of heavy metals to our identified bacteria and underlying the mechanisms of degradation, magnification and or bioconcentration of these heavy metals.

Keywords: Heavy metals, tropical marsh, caribbean, bioindicators, BIOLOG®

INTRODUCTION

Heavy metals (HM) are a unique group of naturally occurring compounds released into the environment naturally or by anthropogenic processes. The increment of urban development are an important cause of significant increments of these toxic pollutants that can pose a risk to ecosystems, and in bioavailable forms can later

constitutes a threat to public health[1]. The World Health Organization has reported heavy metals like Arsenic (As), Cadmium (Cd), Lead (Pb) and Mercury (Hg) as the major contaminants of public health concern [2]. Heavy Metals are components of Earth's crust and are essential nutrients for plants and animals but at trace levels. However, all metals can be harmful in high concentrations and prolonged exposure. Via ingestion HM could cause lung, kidney, liver, digestive tract, and pancreas cancers. It also could cause oxidative cellular stress, respiratory problems, cardiovascular diseases, nervous system toxicity, and kidney damage via inhalation [3].

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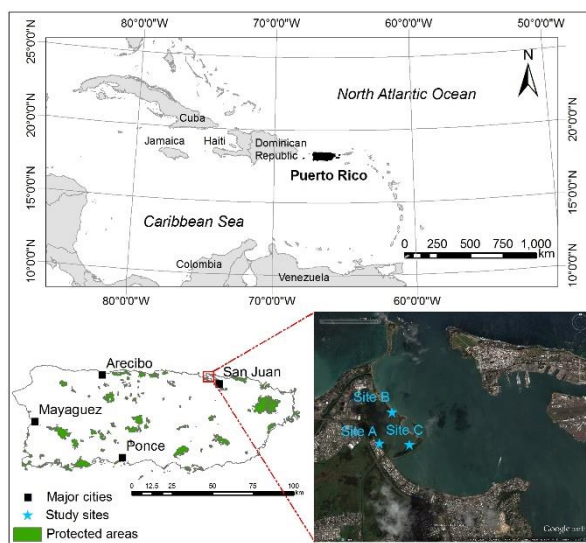


Figure 1. Las Cucharillas Marsh is located in the Northeast coast of the island of Puerto Rico.

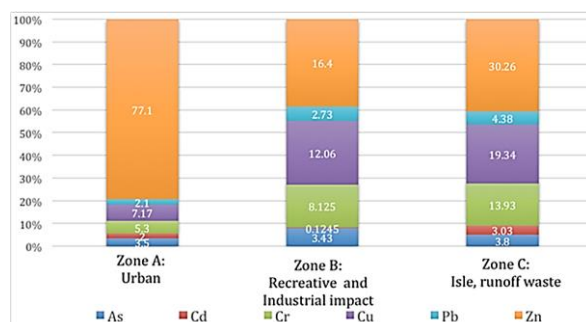


Figure 2. Concentration of metals per area at Las Cucharillas Marsh. The most relevant heavy metals for the study are Arsenic (As), Chromium (Cr), Copper (Cu) and Zinc (Zn). These heavy metals are relevant for our study for their association with Heavy Metals resistant. In Zone A, As (4%), Cr (5%), Cu (9%) and Zn (80%). In Zone B, As (9%), Cr (16%), Cu (28%) and Zn (38%). In Zone C, As (6%), Cr (15%), Cu (25%), and Zn (40%). Adapted from Mejías-Musa Sanco Laboratory [10].

In Puerto Rico, cancer is the second disease that causes the greatest number of premature deaths with 5,000 deaths each year [4]. The Cancer Comprehensive Center of Puerto Rico informed that the incidence of cancer from 2005-2010 was higher for the metropolitan and southeast area of the island. For men the most common cancer is prostate cancer (41%), while for women the breast cancer (30%). For both men and women, the colorectal cancer is on the same proportion (13%). Therefore, the environmental impact due to these pollutants in soil, water, and live organisms is detrimental. The bio-

remediation could be used to restore this tropical ecosystem [5] and contribute at the same time to a better human health. Some pollutants are known to bioconcentrate and/or biomagnify in fish, crabs and other commonly eaten marine organism tissue [6]. We have this concern understanding that Cataño has two fishing villages. One potential method for the bioremediation of the site is using microorganisms. The tolerance of soil bacteria to HM has been proposed as an indicator of the potential toxicity to other forms of biota [7] [8]. The objective of this research was to assess the bacterial community on the mangrove marsh, identifying these bacteria and correlating bacterial species with the type and concentration of the metals found. This way we can have a baseline of what kind of microorganisms inhabits in this kind of environment and how we can use them for future HM assessing purposes.

MATERIALS AND METHODS

Description of study site

Las Cucharillas Marsh is located in the municipality of Cataño near the San Juan Bay portuary zone and covers an approximate area of 1,236 acres (Figure 1) [9]. Our research site is also known as “La Esperanza” Peninsula, located at the following coordinates 18°27'06.28" N and 66°08'07.09" W. Coordinates were calculated using Garmin 72H GPS Technology.

La Esperanza peninsula is composed primarily of herbaceous wetlands, mangroves, and open water areas. This wildlife refuge located on the northeast coast of Puerto Rico (PR) island and is part of the San Juan Bay Estuary (SJBE). The SJBE is composed of interconnected wetlands where three mangrove species coexist; *Rhizophora mangle*, *Laguncularia racemosa*, and *Avicennia germinans*. It also has water bodies that run through eight of the most populated municipalities of the island, which are: Bayamon, Toa Baja, Cataño, and Guaynabo [10]. Previous research had demonstrated that these mangrove species have the capacity of phytoremediation, sequester and detoxify pollutants due to their ability to extract and accumulate heavy metals in the root/sediment interface, and different parts of the plant [10]. Las Cucharillas Marsh is close to many industries, urbanized and recreational areas, and is frequently visited by fishermen, tourists, and families around the year. For purpose of this study, the research location was divided into three main study zones or sites (A, B, and C) (Figure 1) and each zone has specific characteristics. The zone A (18°26.980"N, 66°08'07.203"W) is the most urbanized area and closer to La Malaria Creek. The zone

B (18°27.270"N, 66°08'07.032"W) includes a recreational park and is product of the dredging of the bay. It was reopened in 2006 after hurricane George demolished it in September 1998. The zone B is the closest one to a rum distillery, established in PR in 1936 which produces 85% of worldwide rum [11]. Finally, the zone C (18°27.012"N, 66°08'07.851"W) known as the Esperanza Isle. This isle is serving as a depository for solid waste like, automobile tires, plastics, and biomedical instruments that high ties bring to the shore [10]. As a valuable resource for its ecological, social and cultural services, this estuary has been evaluated since 2000 to become a protected and conserved ecosystem. Nevertheless, the human activities have impacted adversely with uncontrolled urban and industrial development that includes: fishing docks, recreational parks, houses, oil refineries, rum distillery and several thermoelectric factories. Effluents disposal and leachates, from urban and industrial discharges, as well as the atmospheric pollutant deposition, contaminates the marine environment [12]. Another aspect in which the wetland has been degraded is the sanitary discharges due to the absence of sanitary sewers in nearby communities [13]. These anthropogenic sources are highly hazardous to the ecosystem and human health because they could represent the primary causes of heavy metal contamination in wetland's sediment [10]. Some HM that had been associated with anthropogenic activity in the study area includes Cu, Cr, Pb, Zn, Cd and Hg (Figure 2) [13].

Sampling and sediment analysis

Coliform bacteria bacteriological methodology

A total of 33 surface soil samples were extracted following the EPA SOP #EH-02 [14]. The samples were transported properly to a private laboratory for the quantification and identification of bacteria. Multiple tube fermentation test with a selective media and heterotrophic plate count were used to determine fecal and total coliforms in samples. Statistical analysis of these results was performed in Universidad del Este.

Sampling

A total of 60 surface soil samples were extracted with a manual metal auger [15] to ten (10) inches depth, discarding the first inch. After the soil extraction, the auger was sterilized with alcohol and flamed with a manual burner to avoid cross contamination [16]. Physical parameters (pH and Temperature) were measured at the study site using a Kelway HB-2 Soil Acidity & Moisture Tester and a Mercury Lab Thermometer. The samples

were transferred properly to a Nasco Whirl-pak® sterile bags, sealed and refrigerated in a 4°C cooler until lab processing.

Procedure

A homogenization process of the samples was performed manually for one (1) minute [17]. After homogenization one (1) gram of the sediment was added in 100 mL of distilled water. Using the Genie-2 Vortex, the samples were mixed and then settle down for 24 hours at room temperature. The supernatant was filtered using EPA 1600 membrane filtration method with a membrane of 0.45 µm pore after the 24 hour period. Each membrane was plated in four different growth media after the filtration method. All membranes were incubated at 29°C for 18 – 24 hours. After incubation, the CFU was counted. Four colonies were selected by phenotypic characteristics (color, appearance, and distribution) to obtain a representative bacteria population from each plate. Each colony was sub cultivate at the same growth media at 29°C for 18 – 24 hours.

Media

Four growth media were prepared to obtain a representative bacteria population of the sediment sample. MacConkey Agar to grow Gram-Negative Bacteria. Mannitol Salt Agar for selecting members of the genus *Staphylococcus*. The mEndo Agar for identifying several strains of enterobacteria and coliforms. Tryptic Soy Agar for heterotrophic bacteria.

Biolog®: Biochemical identification

After sub-cultivation and purification of the previously selected colonies, another incubation at 29°C for 18-24 hours was needed before the BIOLOG® GEN III protocol to identify the bacteria. Five (5) colonies were identified, the others 55 remains cryogenized in -80°C for subsequent identification.

Statistical analysis

Sediment samples were taken to a local laboratory to determine a bacteriological baseline of the number of bacteria present on the marsh. Data revealed a total of 1790 CFU of total coliform bacteria (TCB) and 996 CFU of fecal coliform bacteria (FCB). The T-Test of Mean Differences revealed a p-value of 0.003 demonstrating a significant difference between the bacterial compositions on the site. Also, a significant statistical difference was observed when the bacteriological concentration was analyzed and compared with sampling area. A p-value of 0.038 was obtained using the T-Test of Mean Differ-

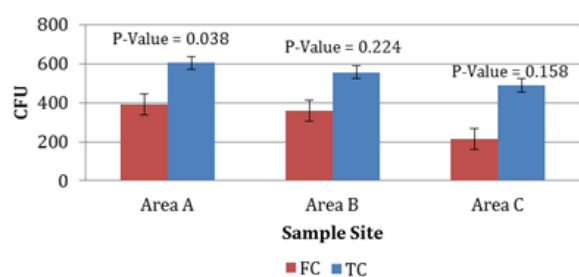


Figure 3. Bacterial Number per Sampling Site. X axis, values in colony forming units. Y axis samples site associated with Las Cucharillas Marsh. Blue color bars represent total coliforms (TC) values and red color bars represented fecal coliforms (FC) values related to the sampling site using heterotrophic plate count methodology.

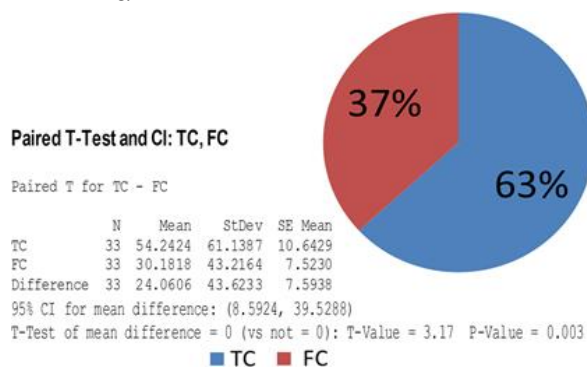


Figure 4. Determination of fecal and total coliforms in Las Cucharillas Marsh Sediments

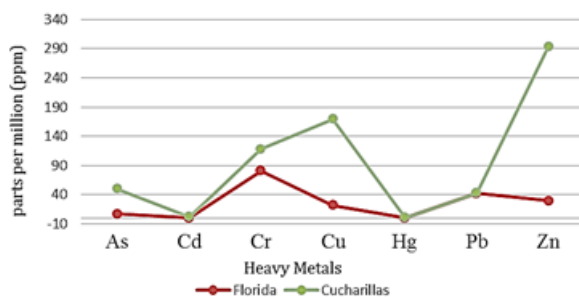


Figure 5. Comparison of highest values for heavy metals found in urban soils. X axis indicates values in parts per million (ppm). Y axis indicates heavy metals found at the highest concentration at Las Cucharillas. Maximum concentration based on the natural background of trace elements from Florida's Baseline in red color [34] - and Maximum concentration (ppm) found at the study site in green color [10].

ences in area A between TCB and FCB.

RESULTS AND DISCUSSION

Coliform Bacteria Bacteriological Analysis – Analy-

sis was performed to compare the soil microbial communities in each zone of Las Cucharillas Wetland. The Heterotrophic Plate Count Technique revealed the presence of coliform bacteria including total and fecal coliforms in soil samples. When species of the coliforms group are present in aquatic and soil environment they become indicators of fecal contamination [18]. Each zone (A, B and C) showed the ubiquity of both groups of coliforms. This data reflects the occurrence of runoff water and illegal sewage water discharge as one of the possible pathogen transport pathways to this wetland. Zone A showed a major distribution of total and fecal coliforms due to urban runoff (Figure 3).

Aqualab laboratories

Among the identified bacteria were: *Escherichia coli*, *Klebsiella* sp. which belongs to the fecal coliform group, and *Pseudomonas* sp. which most of them are antibiotic-resistant specifically to penicillin [19]. However, the proportion of these coliform bacteria through the wetland revealed a major distribution of total coliforms with 63% versus 37% of fecal coliforms. This proportion (Figure 4) demonstrates a diverse bacterial population on the sediment of the wetland. Studies revealed that bacterial communities in salt marshes sediments are vast, and those microorganisms can affect the availability of heavy metals [20].

Biolog® identification

According to the GEN III protocol, the result indicate several metal resistant bacteria: *Ralstonia pickettii* and *Cupriavidus gilardii* (Table 1). Those microorganisms are both Gram-negative, aerobic rods which are commonly isolated from highly heavy metal contaminated soils [21]. Xie et al studied the resistance to heavy metals, such as cadmium (Cd), copper (Cu), and zinc (Zn) of *R. pickettii* specifically. Several studies confirmed that Gram positive and Gram negative bacteria showed heavy metal tolerance against Cd^{2+} and Cu^{2+} [5, 22]. These findings can be related to high concentration of Zn and Cu in the sediment of the marsh (Figure 5).

The heavy metals found in the area were compared with the Florida Baseline for heavy metals in sediment due to the lacking of heavy metals studies in our sampled site. Among those metal resistant bacteria, which were identified with the GEN III protocol used at our laboratory are (2) Gram positive bacteria: *Enterococcus maloduratus* and *Microbacterium flavescens*. This species of *Enterococcus* is an opportunistic pathogen associated with human and animal gastrointestinal tract mi-

Table 1. Biochemical identification identifies some microorganisms: *Ralstonia pickettii*, *Cupriavidus gilardii*, *Microbacterium flavescens*, *Enterococcus maloduratus* and *Carnobacterium inhibens*. Relation of bacteria with our physic-chemical parameters and association with heavy metals are present in one way or another in the bacteria profile.

Species Name	% of ID	HM Associated	Alkaline pH	Hypersaline Environment	Soil Sediment	Specific Characteristics
<i>Ralstonia pickettii</i>	64	x		x	x	Gram Negative. Resistant to Cu, Ni, Fe and Zn. Opportunistic Pathogen.
<i>Cupriavidus gilardii</i>	69	x	x		x	Gram Negative. Resistant to Cu. Human Opportunistic Pathogen
<i>Microbacterium flavescens</i>	80				x	Gram Positive. Found in mammalian intestinal tract and dairy products.
<i>Enterococcus maloduratus</i>	13		x	x		Gram Positive. Cause Nosocomial Infections.
<i>Carnobacterium inhibens</i>	76		x	x		Gram Positive. Facultative anaerobic Rod

* Percent of identification based on phenotypic pattern in BIOLOG GEN III Microassay

croflora [23]. Studies of ecology and epidemiology of *Enterococcus* spp. recognized that this pathogen is not native of environmental sources as soil or water, thus is widely reported as a contamination of animal sources and waste-water pollution [24] in those ecosystems including recreational waters [25]. A distinctive property of this genus is their ability to grow in the presence of salt (6.5% NaCl). Recently studies had discovered that the sediments of fresh and marine water are a new niche for the survival of *Enterococcus* sp. [26], which includes *E. maloduratus*, the species we isolated. The emergence of this microorganism in water poses a risk to health.

The *Enterococcus* sp. can be an antibiotic resistant bacteria linked to nosocomial infections [27]. These organisms can break down the beta-lactamic rings in ampicillin and vancomycin; and in the aminoglycosides group such as the streptomycin and gentamicin [28]. *Microbacterium flavescens* is a scotochromogenic mycobacterium, acid-fast bacillus that is tolerant to 5% w/v of NaCl, positive for Nitrate and has a growth rate fluctuate between 25 – 37°C [29]. An isolated from an AIDS patient that was successfully treated with anti-mycobacterial drugs suggests that this organism was the causative agent of the infection [29]. The presence of another Gram-positive bacteria identified by the GEN III protocol was *Carnobacterium inhibens*. This bacterium are ubiquitous lactic acid bacteria isolated from diverse sources; including live fish, marine sponges, deep sea sediment, and a range of raw foods and dairy products [30]. Furthermore, the distributions of their natural habitats are vast from temperate/polar climate to aquatic and terrestrial environments [31] [30]. Several studies

have isolated *C. inhibens* from two different sources; a horse manure pile [32] and the gastrointestinal tract of the Atlantic salmon (*Salmo solar*) [33]. Some *Carnobacterium* sp. possess many traits of survival like growing at low temperatures it has not been described other physical/chemical parameters like salt content, atmosphere and pH levels survival rates [30]. However, the identification of this *C. inhibens* in an estuarine environment that is highly impacted by heavy metal pollution is a new finding it must remain studied. Microorganisms with the ability to biodegrade, bioremediate or mitigate environmental problems like HM contamination, should be actively pursued as an eco-wise alternative for this site.

CONCLUSION

The lack of appropriate domestic sewage on the urbanized areas: Juana Matos and Puente Blanco, nearest to Las Cucharillas Marsh, revealed the problem of Fecal and Total Coliforms in high concentrations. This insight contributes to the ecological as well as environmental health stressors in the area. Due to the diversity and richness of microbes on site, bacteria could be used as bioindicators of heavy metals concentration, thus, water quality. For example, *R. pickettii* and *C. gilardii* fit the profile for our research. These microbes have shown resistance to Cu, Zn, Fe and Ni.

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