Distribution of polycyclic aromatic hydrocarbons degrading bacteria in southern coastal sediments of Puerto Rico

by

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Abstract

Most probable number technique was used for enumeration of heterotrophic bacteria. For enumeration of PAHs, the degrading bacteria overlayer technique was used. Higher numbers of heterotrophic bacteria were observed for all sampling sites during June and November. During the rainy season, higher numbers of naphthalene-degrading bacteria (1.6 x 10^3 CFU/g) were found in Guayanilla Bay sediments. Possible explanations may be the input of nutrients through the river effluents and mixing of bottom sediments. However, during the dry season, lower number of naphthalene but higher numbers of phenanthrene-degrading bacteria (8.8 x 10^3 CFU/g) were observed in Guayanilla Bay sediments. That may be due to the absence of the growth factors during the dry season that are necessary for growth of naphthalene-degrading bacteria. Low numbers in hydrocarbon-degrading bacteria were observed in Guánica samples during all sampling periods. Bacterial isolates were identified by 16S rDNA as species from the genera: *Alteromonas, Microbulbifer,* and *Vibrio*.

Resumen

El conteo de bacterias heterotróficas se realizó mediante la técnica del número más probable. La técnica de esparcido en plato se utilizó para la enumeración de bacterias degradadoras de hidrocarburos aromáticos policíclicos (HAPs). Durante los meses de junio y noviembre se observó en todas las áreas bajo estudio, alto conteo de bacterias heterotróficas. En el período de lluvia, los sedimentos de la Bahía de Guayanilla presentaron alto conteo de bacterias degradadoras de naftaleno (1.6×10^3 CFU/g). Esto puede ser causado por el aporte de nutrientes a través de los afluentes del río Guayanilla y la resuspensión del sedimento. Durante eventos de sequía, se observó un alto conteo de degradadoras de fenantreno para las muestras de la Bahía de Guayanilla (8.8×10^3 CFU/g). Este aumento puede ser causado por la ausencia de factores de crecimiento durante períodos de sequía que sean requeridos por las bacterias degradadoras de naftaleno. Las bacterias aisladas fueron identificadas mediante el 16S rDNA como *Alteromonas*, *Microbulbifer* y *Vibrio* sp.

I dedicate this work to my grandmother
Monserrate Bermúdez Cuevas, who always has the wisdom to teach me and guide me through life.

Also to all my family, my husband, and my son who were supportive and patient during the course of this work.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

CEER	Center of Energy and Environmental Research
CORCO	Commonwealth Oil Refining Company
GPS	Global Position System
MPN	Most Probable Number
NCBI	National Center for Biotechnology Information
PAHs	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project
TCBS	Thiosulfate Citrate Bile Salts Sucrose
USEPA	United States Environmental Protection Agency

Introduction

In recent years, due to the increase in industrial and human activities, as well as atmospheric deposition from natural sources, pollution levels of polycyclic aromatic hydrocarbons (PAHs) have increased steadily in coastal environments. In 1999, it was estimated that 1.7 and 8.8 x 10^6 tons of petroleum hydrocarbons impacted global marine ecosystems annually (Head and Swannell, 1999). PAHs exhibit carcinogenic, mutagenic, and toxic properties. PAHs are listed by the United States Environmental Protection Agency (USEPA) and European Commission as priority pollutants (Amellal *et al*., 2001). For example, phenanthrene is considered as mild allergen, human skin photosensitizer, and a potent inhibitor of gap junction intercellular communications (Weis *et al*., 1998). PAHs are also considered as persistent organic pollutants resulting in biomagnification in the food chain in the environment, which may adversely affect human health and the biota in the environment (Jong-Su *et al*., 2009).

The major sources of PAHs accumulation in the environment by human activities are by coal gasification, tar oil distillation plants, incomplete fuel combustion process, waste incinerators, and accidental spillage of petroleum products by ships. Urban runoff, industrial wastewater, and rivers are mainly the overland routes to transport these compounds to the marine environment (Yim *et al*., 2005). The hydrophobic properties and high adsorption capabilities of PAHs to organic matter present in the marine sediments may make the marine sediments as major repositories of these compounds.

Most of the industrial cities in Puerto Rico are located near the coast. Thus, coastal environments of Puerto Rico are prime repositories of PAHs because of industrial, urban, and maritime activities. Contamination by PAHs may adversely affect coral reefs and can be toxic to

fish and algae (Rodríguez *et al*., 2007 and Incardona *et al*., 2004). The Guayanilla coast in Puerto Rico was the site of one of the largest petrochemical industries in the world until it was shut down in 1982 due to economic reasons. Higher numbers of PAHs-degrading bacteria were reported in the coastal environment surrounding the petrochemical complex, indicating exposure to higher levels of PAHs (Zaidi and Imam, 2005).

Chemical, physical, and biological methods have been developed to clean up shoreline ecosystems. Microbial remediation (bioremediation) is a potential tool for cleaning up the contaminated coastal sites. Using this technique, it may be possible to bring the concentration of PAHs to acceptable levels. Quality and ecological value of estuarine and marine ecosystems would be improved by remediation techniques. In economic aspects, microbial remediation has been accepted as a cost-effective process compared with other methods used for the same purpose. Bioremediation is the only practical method for the degradation of organic contaminants where the impact on the environment is minimal because bacteria convert toxic compounds into non-toxic or less toxic end products (Arbabi *et al*., 2004). Carbon dioxide, water, and cell biomass are produced by complete biodegradation. To enhance biodegradation, cultured bacteria capable of degrading PAHs are sometimes introduced into the environment. In these situations, isolation and identification of indigenous bacteria that are capable of degrading PAHs under local conditions may eliminate costly practices of using commercial bacteria that may not be successful under local environment conditions (Zaidi and Imam, 1996).

Expansion of Ponce port by dredging to relieve congestion of port in San Juan is now in progress. To our knowledge, there have been no studies of PAHs biodegradation in Ponce Bay sediments and there have been only a few in Guayanilla Bay. It is for this reason that potential for microbial transformation of PAHs by indigenous microbial populations in these sites should

be studied. Evaluating the capacity to biodegrade petroleum derived products in marine sediments samples can determine the degradative potential of indigenous microflora and will help in understanding the fate and transport of these compounds.

Research Objectives

The principal objective of this research was to isolate, identify, and study potential PAHs-degrading bacteria in marine sediments. The specific aims of this research were:

- Use of naphthalene, phenanthrene, and chrysene as model PAHs to determine the degradative potential of the microflora in sediments.
- Determine the number of heterotrophic bacteria in sediment samples.
- Determine the number of PAHs-degrading bacteria in Guayanilla Beach, El Faro, Ponce Bay, and compare their numbers from the relatively uncontaminated site of Guánica Beach, Atolladora, which was used as control site.
- Isolate, purify, and identify PAHs-degrading bacteria in marine sediment samples from Guayanilla Beach, El Faro, Ponce Bay, and Guánica by standard microbiological techniques and substrate utilization tests.
- Use molecular techniques to identify PAHs-degrading bacteria from the sites.

Literature Review

Polyaromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are of environmental concern because of their persistence and worldwide distribution in the environment. The lipophilic natures of PAHs increase their potential for biomagnification in the food chain. PAHs are organic compounds which consist of fused aromatic rings where solubility decreases with increasing molecular weight and salinity (Poeton *et al*., 1999 and Zaidi and Imam, 1999a). Thus, the environmental fate of PAHs will be determined by their molecular size, number of aromatic rings, pattern of ring linkage, availability of sorption to sediment, and biodegradation (Miriam *et al*., 2006 and Poeton *et al*., 1999). The physical properties of PAHs affect their bioavailability and toxicity to freshwater and marine organisms (Neff *et al*., 2005). PAHs represent the largest class of contaminants for which bioremediation was adopted as a treatment action at Superfund sites in the United States (USEPA, 1996).

Sources of PAHs

Accidental spillage of petroleum products, automobiles exhausts, coal gasification, discharge of industrial effluents, fuel combustion, gas production, incomplete combustion of organic materials, waste incineration, and wood treatment facilities are considered the major sources of PAHs into the environment (Cerniglia, 1992, and Jong Su *et al*., 2009) Natural sources of PAHs such as forest fires, mineral production, and thermal geologic reactions, are minor contribution to PAHs contamination (Mrozik *et al*., 2003). PAHs provide the starting materials for the production of many agrochemicals, explosives products, pharmaceuticals, and polymers (Gibson *et al*., 1971). For example, naphthalene and phenanthrene have been used in the synthesis of detergents, dyes, fungicides, and pesticides (Shennan *et al*., 1984). Chrysene is

used as preserving compound in wood treatment facilities. Concentration ratios of anthracene and phenanthrene are used to determine the possible sources of contamination. High Phe/Ant ratios may indicate petrogenic sources (Zheng *et al*., 2004 and Neff *et al*., 2005). Southern California's treatment plants are estimated to discharge more than 17.4 x 10^3 mt of hydrocarbons/yr to coastal waters, representing about 6% of the worldwide input of wastewater-borne petroleum into the ocean (Eganhouse and Kaplan, 1982).

PAHs in sediments

In marine environment, the sediments are the final receivers of PAHs. Highest values of PAHs concentrations are recorded in estuaries and coastal areas, areas with intense vessels transport, and oil treatment (Nikolaou *et al*., 2009). Accumulation of PAHs can be significant in harbors where renewal of water with the open sea is limited. Concentrations of PAHs from 9 to 31,774 ng/g/L were recorded in Naples harbor (Sprovieri *et al*., 2007). On the other hand, Woodhead *et al*. (1999) found in estuaries sediment samples in England PAHs values greater than 10,000 µg/kg dry weight. Individual concentrations of PAHs were detected from 100 to 1000 µg/kg dry weight. Studies conducted in Mediterranean coast revealed high concentrations of PAHs in marine sediments, but the overlying waters were considered clean (Telli-Karakoç *et al*., 2002). In mangrove swamps, concentrations of total PAHs ranged from 169 to 1,058 ng/g dry weights in tropical coasts of Hong Kong (Guo *et al*., 2005). High concentrations of phenanthrene are found in PAH contaminated sediments, wastes sites, and surface soil (Moody *et al*., 2001). Two to four ring PAHs were found in sediment samples of Naples harbor with median concentration values of 60-70% of the total PAHs concentrations (Telli-Karakoç *et al*., 2002 and Nikolaou *et al*., 2009).

PAHs as model compounds

Low molecular weight PAHs such as naphthalene and phenanthrene are used as model compounds to study PAHs degradation. The simplicity and relatively high solubility of naphthalene is useful for bacterial degradation studies (Mrozik *et al*., 2003). Naphthalene is considered a common micro-pollutant in potable water (Samanta *et al*., 2002). Phenanthrene is a three aromatic ring hydrocarbon found as an intermediary product during biodegradation of petroleum (Zaidi and Imam, 1999). Few studies were conducted using chrysene. Chrysene is a high molecular hydrophobic PAH with carcinogenic properties (Miriam *et al*., 2006).

Health risk

Carcinogenic and teratogenic behavior of PAHs compounds have been observed in animals. Accumulation of PAHs in marine organisms may induce tumors in fish livers, induction of cytochrome P450 detoxification enzymes, affect the immune system and disrupt the endocrine system (Woodhead *et al*., 1999). Narcosis with PAHs in fishes may result in reduction in locomotion performance (Gonçalves *et al*., 2008). Toxicity test with sand dollar embryos demonstrated a 100% of mortality when exposed to sediment samples containing 33.6 and 37.0 µg/g of total PAHs (Meador, 1990). A reduction in larval motility in the coral reefs is expressed by photo-induced toxicity of PAHs, (Peachey and Crosby, 1996). Studies in coral reefs in southwest of Puerto Rico indicated a lower coral species richness when high concentrations of PAHs are present (Pait *et al.*, 2008). Concentration of 25 µg/g of PAHs in urban runoff sediments is considered as moderately toxic for benthic fauna (Neff *et al*., 2005). Total PAHs concentrations were 1000 times higher in mussel samples than seawater samples (Telli-Karakoç *et al*., 2002).

PAHs-degrading bacteria

Worldwide distribution of marine PAHs degraders suggests that the capacity for PAH degradation in polluted environments depends on the diversity and characteristics of naturally occurring populations and response to environmental conditions, rather than on the introduction of new taxa or selective modification of existing bacteria (Chung and King, 2001). The predominant hydrocarbon-degrading element in marine ecosystems of microbial communities is the marine bacteria (Leahy and Colwell, 1990). Several reports have mentioned the isolation and identification of PAHs-degrading bacteria from different contaminated environments. Some PAHs-degrading bacteria isolated from marine environments belongs to the genus *Alteromonas, Alcanivorax, Cycloclasticus, Halomonas, Marinobacter, Marinomonas, Moraxella, Neptunomonas, Oleiphilus, Planococcus, Pseudoalteromonas, Pseudomonas,* and *Vibrio,* (Wong *et al*., 2002 and Harayama *et al*., 2004). *Paracoccus*, *Rhodococcus*, and *Sphingomonas* are isolated from PAHs-contaminated sediments in mangrove swamps (Guo *et al.*, 2005). Berardesco *et al*. (1998) identified *Burkholderia*, *Flavobacter*, and *Mycobacterium* as phenanthrene bacteria degraders in intertidal sediments of Boston Harbor.

Biodegradation

The biodegradative routes of low molecular weight hydrocarbons like naphthalene and phenanthrene have been reported for several bacterial strains (Mrozik *et al*., 2003 and Jong Su *et al*., 2009). The enzymatic capability of microorganisms to biodegrade organic pollutants involves the oxidation of the substrates by oxygenases in the presence of oxygen. Depending of the chemical structure of the compound, some PAHs are transformed to carboxylic acids and others are hydoxylated to form diols (Atlas, 1995). An important feature in biotransformation of organic pollutants is the conformation structure (*cis*- or *trans*-) in its intermediary structures.

Biodegradation by bacteria form *cis*- structures that result in detoxification and the production of non-toxic end products such as carbon dioxide, cell biomass, and water which can be assimilated safely by the food web (Atlas, 1995). Use of other degrading organisms, such as fungi, for bioremediation, form *trans*- structures that have carcinogenic properties that can affect marine biota. The abundance of hydrocarbon-degrading bacteria in the marine environment and their complete biodegradation allow us to safety use these organisms in bioremediation strategies.

Some environmental factors such as light intensity, nutrient availability, oxygen concentration, pH, presence of co-substrates, salinity, season, sediment properties, solubility of pollutants, and temperature can affect the extent and rate of biodegradation (Mrozik *et al*., 2003 and Rowland *et al*., 2000). Poeton *et al*. (1999) found higher rates of PAHs degradation when the bacterial biomass was accompanied with sediment samples. The biodegradation rates of low molecular weight PAHs may be related to total organic carbon content in subtidal marine environments (Hinga, 2003). Enhanced PAHs degradation can be observed in subsurface sediments by burrowing organisms that improve the introduction of oxygen into sediments (Chung *et al*., 2001).

The introduction of hydrocarbons in uncontaminated sites perturbs the local selective conditions, inducing changes in adaptive response, composition, and structure in a microbial community (Ridgway *et al*., 1990). Several methods are used to isolate and identify pollutant-degrading bacteria. A method based on spraying ethanolic solution of phenanthrene on seawater before and after incubation was used to isolate hydrocarbon-degrading bacteria (Berardesco *et al*., 1998). Another method for the cultivation of hydrocarbon-degrading bacteria safely is the overlayer technique, where they can easily be identified by the clearing zone formed around the colony, indicating the use of the substrate by the bacterium (Bogardt and Hemmingsen, 1992).

PAHs studies in Puerto Rico

Few studies in pollutant concentrations and biodegradation are reported from Puerto Rico. In the southwest coast of Puerto Rico, elevated concentrations of total PAHs were found in sediments adjacent to La Parguera, Lajas. Sediment samples collected near agricultural and industrial areas in the Guánica Bay area showed 583 ng/g and 911 ng/g of total PAHs concentrations (Pait *et al*., 2008). Adsorption of PAHs to marine sediments is highly influenced by grain size. According to Pait et al. (2008), automobile emissions may be an important source of PAHs to the town of La Parguera, Puerto Rico. Bioremediation and microbial diversity study was conducted in hydrocarbon contaminated aquifer in Vega Baja. Bacterial isolates belonging to the *Proteobacteria* subdivision (alpha, beta, and gamma), *Bacilli*, and *Actinobacteria* groups were isolated and utilized in the bioremediation study (Rodríguez *et al*., 2006).

In Guayanilla, CORCO refinery processed a wide range of crude and unfinished oil for the production of diesel fuel, gasoline, jet fuel, kerosene, and others (Lair *et al.*,1971). Petrochemical residues are still present in Guayanilla Bay sediments. In 1979, 15 ug/L, and 389 ug/L of petroleum hydrocarbons were found in coastal waters of Guayanilla Bay (López, 1979). Also, high concentrations of metals were found in Guayanilla Bay sediments. In Guayanilla Bay, a few studies were conducted to determine the effects of non-biotic factors such as pH, nutrients, and surfactants on the extent and rate of PAHs biodegradation (Zaidi and Imam, 1999a, Zaidi *et al*., 2003, Rodríguez *et al*., 2007). The addition of nitrogen enhanced the degradation of phenanthrene in Guayanilla water samples, but phosphorus was not a limiting factor (Zaidi and Imam, 1999a). The residence time of PAHs can increase under alkaline conditions and lack of available nitrogen. A gram-negative bacterium identified as *Alteromonas* sp. capable of degrading phenanthrene was isolated from coastal water of Guayanilla Bay (Zaidi

et. al., 1999b). However, to our knowledge no information is available on isolation of PAHs-degrading bacteria from coastal sediments in Puerto Rico.

After the Exxon Valdez oil spill in Alaska, bioremediation by introducing PAH degrading bacterial strains into the environment was shown to be successful. Numerous companies now sell cultures that claim to accelerate biodegradation. However, in tropical areas, indigenous *p*-nitrophenol degrading bacterial strains isolated from the local environment successfully accelerated the degradation of the toxic chemical when introduced into industrial wastewater while inoculation of *p*-nitrophenol-degrading non-indigenous bacteria mostly failed (Zaidi and Imam, 1996). There are several reported cases of accidental oil spills in Puerto Rican coastal waters. Therefore, it is important to isolate and identify PAHs-degrading bacteria from coastal environment of Puerto Rico.

Materials and Methods

Site description

Sediment sampling sites are located in the south coast of Puerto Rico. Satellite locations of sampling sites were as follows: Guayanilla Bay (18^0 00' 24.44'' N, 66^0 46' 03.96'' W), El Faro (17^0 59' 50.14'' N, 66^0 47' 00.28'' W), Ponce Bay (17^0 58' 44.61'' N, 66^0 37' 11.30'' W), and Guánica (17^0 57' 16.33'' N, 66^0 51' 08.96'' W).

Guayanilla Bay

Guayanilla Bay is located on the south coast of Puerto Rico (Fig. 1). More than a quarter century ago, this Bay was the site of one of the largest petrochemical complexes in the world until they were shut down. The Bay is divided in three major zones:

Eastern Bay. The innermost portion of Eastern Bay provided intake water for a power generating plant and to petrochemical industries which were mostly closed during the 1980s. The Eastern Bay is divided in three sub-Bays; Intake Area, Thermal Cove, and Southeast Bay. The Intake Area has the intakes facilities for both the power generating plant cooling system and the former CORCO petrochemical complex. The Thermal Cove area is totally enclosed, except for a narrow mouth. This area receives the thermally-enriched water that has passed through the heat exchangers of the power plant (Goldman, 1978). The Southeast Bay receives the thermally enriched effluents from the Thermal Cove.

Central Bay. This zone contains most of the water in Guayanilla Bay and is exposed to the open waters of the Caribbean Sea. It is about one third the area of the Bay. Guayanilla River empties into this deep Bay that is navigable by large ships and is also the site of port facilities. Dock facilities for raw materials and processed petroleum products for CORCO

are located in this area. A small fishing village, Guayanilla beach, is located in the northern part of the Central Bay. Sediment samples were collected in this part of the Bay. The Bay water receives raw sewage from the fishing village, hydrocarbons and heavy metals released during operation of the petrochemical industries, as well as heated water from the thermoelectric generating plant (Rigau and Sardina, 1980). Recently a gas-based power generating plant namely Eco Electric started operation in this area also.

Western Bay. This area is bounded to the east by the central Bay and by land to the west and north. Because of strong winds, the surface water moves with the wind most of the time. Two sub-Bays, a northern and a southern compose this area. The sub-Bays are separated by a peninsula, which contain the mouth of the Yauco River.

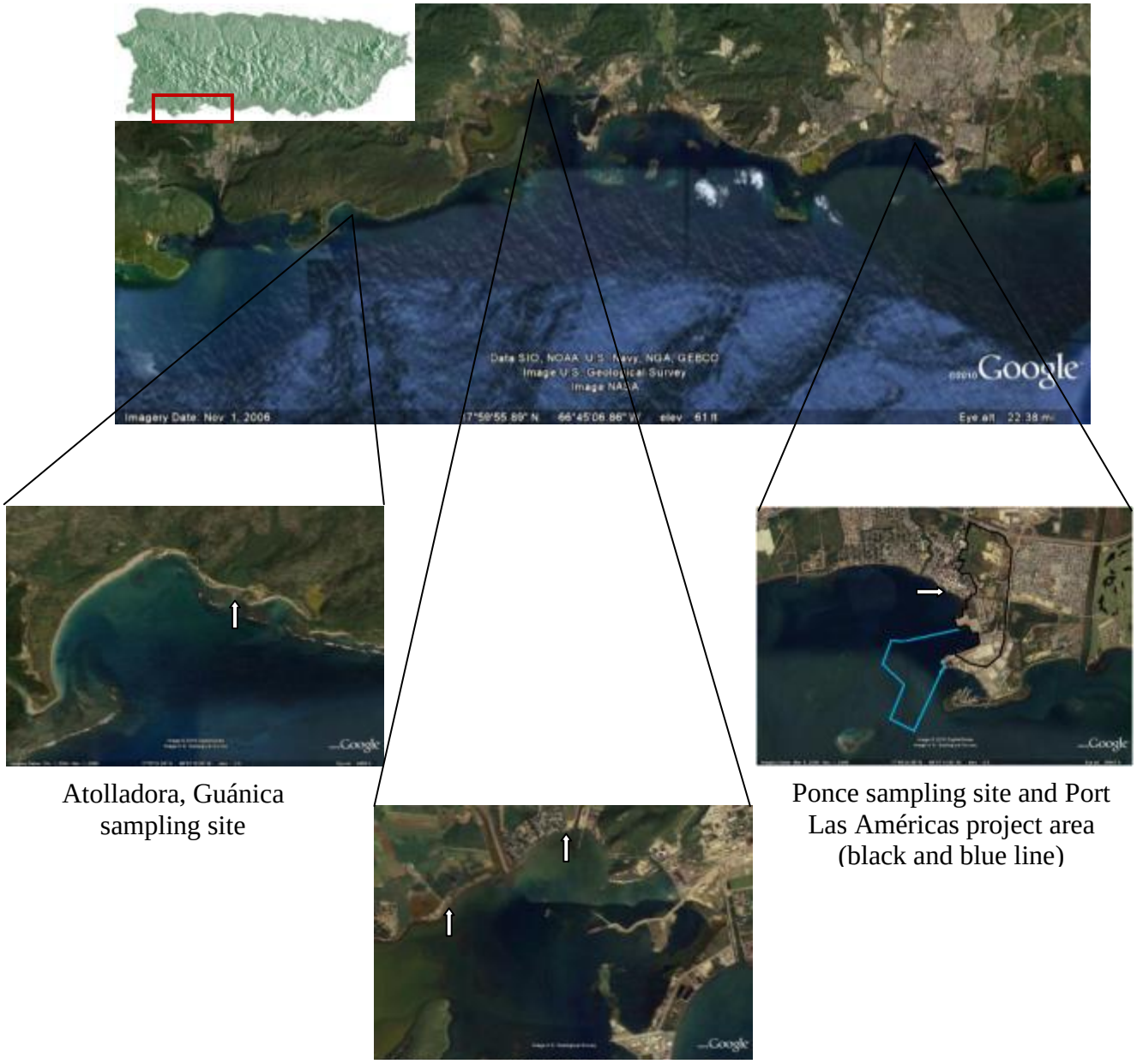
In Guayanilla Bay, diurnal tidal ranges fluctuate from 15 to 45 cm (EQB, 1972). In 1980 Chartock calculated a total tidal exchange of 2.65×10^6 m^3/day, whereas the wind driven flows into the Bay was estimated at 2.92×10^6 m^3/day and 1.95×10^6 m^3/day in and out of the Bay respectively. The net effect of the wind is to transport surface waters into the Bay, whereas the tidal transport of subsurface waters is out of the Bay (Chartock, 1980). Dominant tidal current patterns and easterly winds force waste streams to Punta Guayanilla. The water enters Guayanilla Bay during the flood tide from the open sea and Tallaboa Bay. Winds pattern results in westward drift currents directed toward Guayanilla Bay (Lair *et. al*; 1971). Daily and seasonal factors may control upwelling and the exchange of water in Guayanilla Bay (Chartock, 1980). Hydrologic budgets of the sub-Bays may be considerably affected by the south coast thermoelectric power station.

Ponce Bay

Ponce Bay is located on the south central coast between Punta Cucharas to the west and Punta Carenero to the east. Matilde River and Portugués River discharge water into the Bay (García, 2003). Ponce beach is located to the east of Portugués River, where samples for this study were collected. Cardona Island reef system is located at the entrance of the Bay, whereas Las Hojitas reef is located in the center of the Bay. Cayo Arenas, Cayo Ratones and Cayo Viejo are other reef systems found in Ponce Bay. Prevalent wind patterns in Ponce Bay are from the east-southeast direction. During the late 1990's, Ponce Bay was primarily impacted by waste disposal from a tuna factory and primary-treated domestic sewage plant. Ponce Bay has recently been dredged to make it a deep water "super-port", capable of handling larger ships and alleviating congestion at the San Juan port. The dredging phase of the work was completed in December 2009.

Guánica

The Atolladora area is located near the eastern part of Ballenas Bay; land to the north is known as the Guánica Dry Forest Biosphere Reserve. Guánica is comparatively less contaminated site and is therefore used as a control.



Atolladora, Guánica sampling site

Guayanilla Bay and El Faro sampling sites

Ponce sampling site and Port Las Américas project area (black and blue line)

Figure 1: Satellite map showing sampling sites (arrows) in the south coast of Puerto Rico.

Atolladora, Guánica sampling site.

Guayanilla Bay sampling site.

El Faro, Guayanilla sampling site.

Ponce Bay sampling site.

Figure 2: Sampling sites in Guánica, Guayanilla Bay, El Faro, and Ponce Bay.

Table 1: Evidence of oil spills in sampling sites (in reverse chronology).

Record Number	Date	Location of incident	Quantity	Material Involved
909569	June 24, 2009	Peñuelas	7949.36 L	Oil: diesel
914641	August 12, 2009	Guayanilla Bay	7570.82 L	Bunker oil
905661	May 15, 2009	Guayanilla	189.27 L	Waste oil
914918	August 14, 2009	Peñuelas	378.54 L	Oil: diesel
925635	December 09, 2009	Guayanilla Bay, Dock #1	94.64 L	Oil, Fuel: No. 6
926942	December 23, 2009	PREPA Facility, Guayanilla	1135.62 L	Oil, Fuel: No. 6
866601	March 31, 2008	Guayanilla	Unknown	Oil, Fuel: No. 6
867088	April 05, 2008	Ponce	Unknown	Unknown
869474	April 30, 2008	Peñuelas	794936.48 L	Oil: diesel
874166	June 14, 2008	Ponce, Pier 8	Unknown	Unknown
874172	June 14, 2008	Ponce, Port	Unknown	Unknown
891129	November 27, 2008	Guayanilla Bay, CORCO	Unknown	Unknown
831579	April 07, 2007	Guánica, Playa Santa Beach	757.08 L	Oil: diesel
850837	October 05, 2007	Peñuelas, Tallaboa Bay	189.27 L	Unknown oil
851678	October 15, 2007	Guánica, Ensenada Bay	189.27 L	Oil: diesel
856043	December 01, 2007	Guayanilla, discharge canal area	52049.41 L	Sulfuric Acid
847322	August 30, 2007	Guayanilla Bay to Guanica Bay	Unknown	Unknown oil
850831	October 05, 2007	Peñuelas	Unknown	Unknown oil
852359	October 22, 2007	Guayanilla Bay	Unknown	Unknown oil
856081	December 02, 2007	Guayanilla-Peñuelas	Unknown	Unknown oil
806851	August 06, 2006	Ponce, Cardona Island	3406.87 L	Oil: diesel
815375	October 19, 2006	Guayanilla Bay, Dock #2	Unknown	Unknown
820948	December 14, 2006	Peñuelas	794936.48 L	Oil, Fuel: No.1-D
752000	March 07, 2005	Peñuelas	567.81 L	Gasoline
754946	April 06, 2005	Peñuelas	7570.82 L	Oil: Fuel: No.2-D
774238	September 28, 2005	Peñuelas	378.54 L	Oil: Fuel: No.2-D
779538	November 14, 2005	Peñuelas	757.08 L	Gasoline
780107	November 19, 2005	Guayanilla	Unknown	Oil: diesel
783609	December 28, 2005	Peñuelas	5299.58 L	Gasoline
719801	April 24, 2004	Guayanilla Bay, Intake area	Unknown	Unknown oil
737621	October 07, 2004	Peñuelas, Puntilla Dock	529.96 L	Oil: diesel

From National Response Center Database

Sampling strategy

Sediment samples were collected during eight months in Atolladora, Guánica; Guayanilla Bay; El Faro; and Ponce Bay. Four samples corresponded to dry months (December 2008, March, April, and May 2009) and four samples correspond to the rainy months (January, June, October, and November 2009). For Guánica sample, June 2009 correspond to dry month. Samples were taken in the morning to minimize the effects of wind, tides, and temperature.

Sample collection

Sediment samples were collected in wide-mouth amber glass bottles. The amber bottles were washed with 0.005 N hydrochloric acid (HCl) and rinsed three times with distilled water. The sample bottles were washed with seawater prior to sampling. Sediment samples were transported on ice and analyzed within 24 hr.

Sediment quality parameters

Temperature measurements were routinely measured with a bimetal thermometer. Salinity was determined with a Sper Scientific refractometer (model 300011) with automatic temperature control (ATC). The pH was determined using a pH meter with automatic temperature control (LaMotte Instruments). Determination of ammonia, nitrite, nitrate, and total phosphorus were analyzed only for sediment samples on December 2008 by Alchem Laboratory (Ponce, P.R.).

Enumeration of heterotrophic bacteria

Enumeration of heterotrophic bacteria was done by the most probable number (MPN) technique as described by Woomer (1996). December 2008, June, and November 2009 samples were used for enumeration of heterotrophic bacteria by this technique. A 1-g sediment sample was placed in 9 mL of 0.22 µm filtered-sterile seawater from the sampling site and vortexed for

60 sec. Serial dilutions (10^{-1} to 10^{-5}) were made and then allowed to settle for 60 sec. Marine agar (Difco Laboratories) plates were divided into five sections for each dilution. Five replicates of 5 µL of each dilution sample were incubated at room temperature for 48 hr. Bacterial growth in each inoculation zone was interpreted as a positive result. An MPN table was used to obtain estimated population in a gram of sediment/mL by multiplying the volume factor by 200 and the dilution factor by 10 (See Appendix 3).

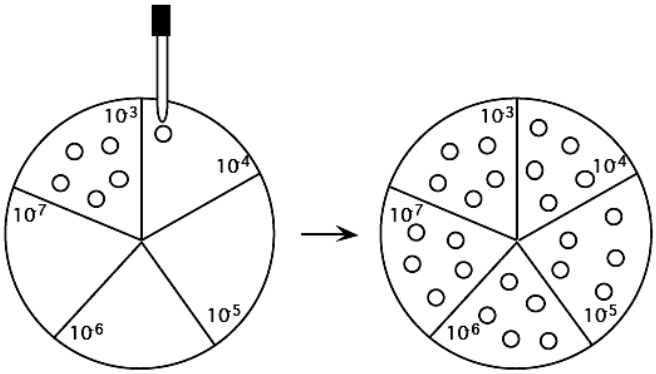


Figure 3: MPN diagram for heterotrophic bacteria enumeration (Fuentes and Massol, 1996).

Enumeration of hydrocarbon-degrading bacteria

Enumeration of hydrocarbon-degrading bacteria was done by modification of the overlayer technique described by Bogardt and Hemmingsen (1992) and Zaidi *et al*. (1999). Modifications to this technique were required because the low numbers of hydrocarbon-degrading bacteria observed mainly in Guayanilla Bay and Guayanilla El Faro sediment samples. A 5 g sediment sample in 9 mL of 0.22 µm filtered-seawater were mixed in a Qorpak beaker and placed in a horizontal position in a rotary shaker (C1 Platform Shaker, New Bunswick Scientific) at 80 rpm for 30 min at room temperature. Sediment samples were allowed to settle for 60 sec and serial dilutions were made.

The pour plate technique with an underlayer of mineral medium was prepared by adding 1.5% of Noble agar (Difco Laboratories) to 300 mL of filtered seawater (0.22 µm) from each sampling site. The solution was autoclaved at 121^0C for 15 min and cooled to approximately 60^oC. Sterile solution of vitamins (0.01 mL) and Bushnell Haas solution (0.25 mL) were added to the medium and poured into disposable 100 x 15 mm Petri-dishes. Stock solution of vitamins contained 2 mg biotin, 5 mg D-calcium pantothenate, 2 mg folic acid, 20 mg niacin, 2 mg *para*-aminobenzoic acid, 3 mg pyridoxal HCl, 5 mg riboflavin, 5 mg thiamine HCl, 5 mg vitamin B_{12} in 100 mL of distilled water. A stock solution of Bushnell Haas was prepared using 0.326 g in 10 mL of distilled water and adjusting the pH to 8.2-8.4. To control fungal growth, 1.12 mL of cyclohexamide at 0.01% dissolved in acetone was added to the underlayer medium, which was adjusted to a pH of 7.8-8.0 with NaOH.

The agarose overlayer was prepared with 80 mL of filtered seawater from each sampling site with 1% of agarose (Fisher Corp.). Then, 3.5 mL of the solution were poured in the tubes and autoclaved. Ethanolic solution (0.25 mM) of the polycyclic hydrocarbon compound to be tested was added to the agarose overlayer. This solution was vortexed for 30 sec and allowed to cool to 30^0C. The sediment sample (0.1 mL) was added to the agarose solution, vortexed, and poured quickly over the mineral medium underlayer. After 3 to 5 d of incubation at room temperature, plates were inspected for colony growth. A clearing zone around the colonies indicated utilization of the model test PAH compound.

Chrysene (0.10%,), naphthalene (0.11%), and phenanthrene (0.15%), with a final concentration of 0.25 mM were used as model compounds to determine enumeration of hydrocarbon-degrading bacteria. An ethanolic solution of hydrocarbon compounds (0.1 mL for naphthalene and phenanthrene and 0.2 mL for chrysene) was added into the agarose overlayer.

Procedures for optimization for the enumeration of hydrocarbon-degrading bacteria

The following modifications were tried to maximize the enumeration of hydrocarbon-degrading bacteria:

(1) After October 2006, instead of one gram of sediment in 9 mL of seawater, 5 grams of sediments were added to 9 mL of seawater to increase the recovery of hydrocarbon bacteria.

(2) For preparation of the underlayer, the following modifications were tried.

   a. Use of seawater from corresponding sampling site

   b. Use of artificial seawater (Cl to 19 ‰) described by McClendon *et. al.* (1917).

   c. Artificial seawater with and without vitamins (1 µL per 1 mL of medium). Vitamins (10 µL) and trace elements solution (1.5 mL) was added to agarose overlayer. Trace elements solution was prepared as described by Bogardt and Hemmingsen (1992).

(3) Initially, 0.50 µL Bushnell Haas stock solution was added to agarose overlayer. Preparation of stock solution was necessary to avoid media precipitation. Adjustment of pH (7.8-8.0) was necessary when Bushnell Haas is introduced into the medium.

(4) Initially phenanthrene was used at 2.57 mM concentration. Because the solubility of chrysene was low, hydrocarbons were prepared at 0.25 mM concentration.

Statistical analysis

Statistical analysis was performed using Two-way ANOVA with interaction mode in Minitab program (Appendix 1). Enumerations of PAHs-degrading bacteria were compared from each study site, and sampling period.

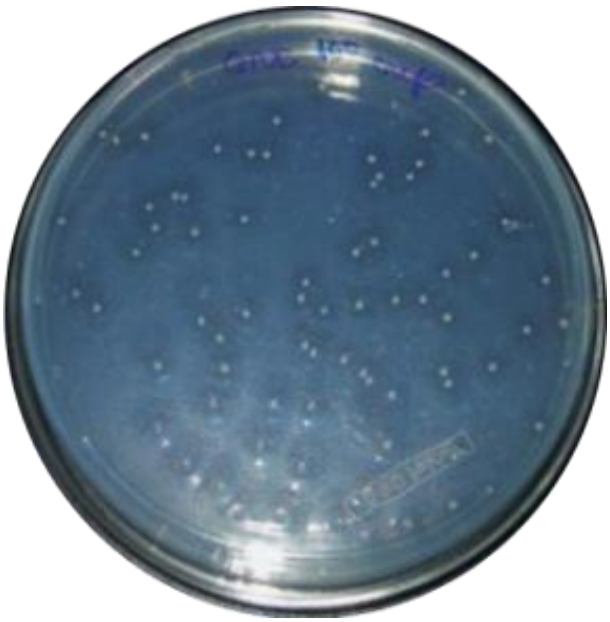


Figure 4: Colonies growing in chrysene at 0.25mM in Guánica sediment samples.

Biolog MT microplates

Microbial degradation of chrysene (8.6 µL), naphthalene (4.3 µL), and phenanthrene (4.3 µL) at 0.25 mM was evaluated in Biolog MT microplates. Each microplate well contained 100 µL of sample and 500 µL of seawater with Bushnell Haas. Microplates were incubated at room temperature for 3 days and changes in color were recorded. Control 1 consisted in 150 µL of sample and 50 µL seawater containing Bushnell Haas. Control 2 was seawater with Bushnell Haas only.

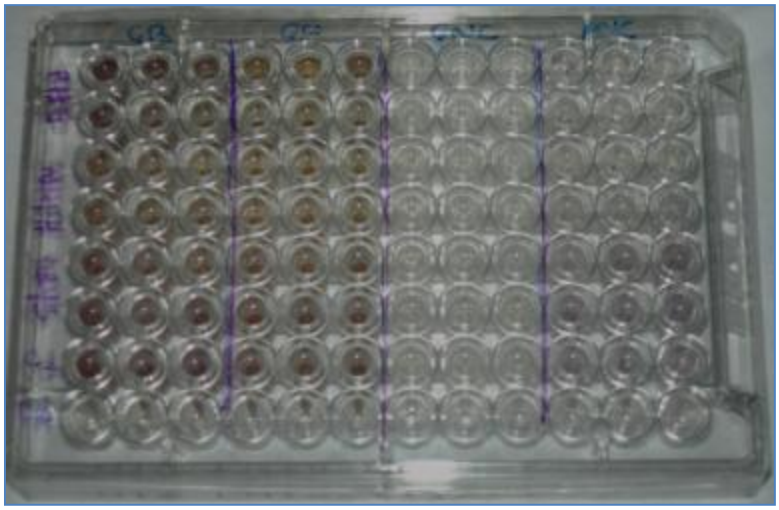


Figure 5: Biolog MT microplates inoculated with sediment samples of each study site and 0.25 mM of hydrocarbon compounds.

Isolation, purification, and identification of PAH-degrading bacteria

Isolation and purification of PAH-degrading bacteria

Bacterial growth in plates with hydrocarbon as the sole carbon source is indicated by a halo of clearing around each colony (Fig. 3). Discrete colonies were isolated and further purified by repetitive plating in marine agar. These bacteria were retested for their ability to degrade the hydrocarbon compound. The isolated strains were characterized based on their phenotypic characteristics.

Morphological and physiological tests

Cultures isolated from the overlayer technique were examined according its macroscopic morphological characteristics. Color, texture, and shape of their colonies were evaluated in

marine agar medium. Their morphology and gram reaction were evaluated by light microscopy. MacConkey medium and TCBS medium were used to determine fermentative capabilities.

Substrate utilization pattern

Substrate utilization patterns of the isolated strains were performed for arabinose, arginine, sorbose, manitol, methyl α-D-glucopyranoside, ribose and sucrose (Manero and Blanch, 1999). In each well, 125 µL of sterile saline phosphate buffer (0.85 g NaCl, 0.065 g K_2HPO_4, and 0.035 g KH_2PO_4 dissolved in 100 mL of distilled water) were added in 96-well microtiter plates. A mineral medium (0.12 g KH_2PO_4, 0.12 g NH_4NO_3, 0.61 g NaCl, 0.024 g $MgSO_4 \cdot 7\ H_2O$, and 0.024 g yeast extract per 100 mL distilled water) at 7.4 pH was divided in 90 mL aliquots for each carbohydrate. To each aliquot, 1.1 g of a carbohydrate was added and pH was adjusted to 7.4. The solution was filter sterilized through 0.22 µm filter, dispensed in Corning bottles and placed into a 50^0C water bath. A solution of 2.5 g of agar with 0.04 g of bromothymol blue (Sigma Laboratories) were prepared in 100 mL of distilled water and 10 mL of this solution were added to each carbohydrate mineral medium. Aliquots of 150 µL of each medium were added to microtiter plates.

Bacterial isolates incubated in microtiter plates with marine broth were transferred with a replicator to the saline phosphate buffer plate and then transferred with fresh replicators to each corresponding plate containing the substrate to be tested. Plates were sealed with paraffin and incubated at room temperature for 72 hr. Results were recorded as positive if the well changed color from blue to yellow and negative if there were no color changes.

Identification of bacterial strain with molecular techniques

DNA fingerprinting of bacterial isolates

Hydrocarbon-degrading bacteria were further characterized by partial 16S rRNA gene sequence analysis. Isolates were incubated in marine broth at room temperature for 48 hr. DNA was extracted from cells using lysis buffer (1.0 mM EDTA pH 8.0, 1% SDS, 20 mM sodium-acetate pH 8.0, and 40 mM Tris-acetate pH 7.8-8.0), and lysozyme treatment (10 mg/mL lysozyme, Tris-HCl pH 8.0 at 37^0C). For DNA precipitation, sodium chloride (5M) and absolute ethanol were used; recovery of DNA was made by resuspending in 50 µL of TE Buffer at pH 8.0 (Saano *et al*., 1995). Genomic DNA was stained with ethidium bromide; 0.8% agarose was used to determine DNA quality.

The bacterial DNA was used as a template for the amplification of the 16S ribosomal DNA gene using the following universal primers (Promega Corporation): forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1392R (5'-ACGGGCGGTGTGTACA-3'). PCR for the 25 µL reaction volume was conducted with 1 µL of DNA template and 6.5 µL nuclease- free water, 12.5 µL of PCR Master Mix (Promega Corporation), and 2.5 µL of 16S rDNA primer [10 pmol/µL]. The cycling parameters used for PCR reaction were: denaturation at 94°C for 3 min, followed by 30 cycles of melting at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 3 min and a final extension at 72°C for 10 min. PCR amplicons were purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation) and product concentration was determined with Hind III markers in 1% of agarose gel. PCR products were sent to Nevada Genomics Center at University of Nevada. Sample preparation was according to Nevada Genomics Center requirements (http://www.ag.unr.edu/genomics/).

Phylogenetic analysis

The sequence of 16S rRNA gene obtained was compared using *Seqmatch* and *Classifier* from the Ribosomal Data Project II (http://rdp.cme.msu.edu/) and sequences were edited in Note Tab Light editor program and with the BioEdit Sequence Alignment Editor Software (Wang *et al*., 2007). Mega 5 software was used to sequence alignment by ClustalW (Tamura *et al*., 2007); the neighbor-joining method with *p*-distance values was used to assemble a phylogenetic tree.

Results

Sediment quality parameters

Measurements of temperature, salinity, and pH are shown in Table 2. The temperatures ranged from 22.0^{o}C to 24.0^{o}C from December 2008 to May 2009 sampling periods. Higher temperatures (30.0^{o}C) were observed in all sampling stations during June 2009 with a maximum temperature of 31.0^{o}C for Guánica samples. In October 2009 samples the maximum temperature was 32.0^{o}C in Guayanilla El Faro, followed by Ponce Bay with a temperature of 31.0^{o}C. During November 2009, temperature values ranged from 28.0^{o}C (Ponce Bay and Guánica) to 29.0^{o}C (Guayanilla Bay and El Faro).

Salinity measurements ranged from 31.0 ppt to 39.0 ppt. Constant values of 36.0 ppt were recorded for Guayanilla Bay during all sampling periods. Guayanilla El Faro had salinity of 34.0 ppt during December 2008, October 2009, and November 2009. However, the highest value of 39.0 ppt for this sampling site was recorded in March 2009. During May to June 2009, salinity values were 36.0 ppt and in January and April 2009, salinity was 37.0 ppt. In Ponce Bay, salinity ranged from a low of 31.0 ppt in January 2009 samples to a high of 37.0 ppt in November 2009 samples. Small variations in Guánica salinity measurements were observed, with high of 37.0 ppt in October, and April 2009 samples. The rest of Guánica samples the salinity ranged between 35.0-36.0 ppt.

The pH values in Guayanilla Bay ranged from a low of 7.4 in June 2009 to high of 8.0 in October 2009 samples. The rest of the samples pH values ranged from 7.5-7.7. At the El Faro sampling site, pH values were generally higher than in Guayanilla Beach with three samples (March, April, and October 2009) with high pH values of 8.1. The rest of El Faro samples had pH values between 7.5-7.9. In Ponce Bay, the highest pH of 8.2 was found in October 2009

sample. Slightly higher values of pH were generally observed in Guánica ranging from a high of 8.2 in October 2009 to low of 7.7 in June 2009 sample (Table 2).

The concentrations of ammonia, nitrite, nitrate, and total phosphorus in sampling sites sediments during December 2008 were determined (Alchem Laboratories, Ponce P.R). The ammonia concentration was 5 mg/L in all samples. Nitrite concentration (10.5 mg/L) was slightly higher in El Faro sediments compared to Guánica, Guayanilla Bay, and Ponce Bay sediments (<10.0 mg/L). A higher concentration of nitrate was found in El Faro (42.0 mg/L), while in Guánica, Guayanilla Bay, and Ponce Bay sediments nitrate concentrations ranged from 10.2 to 13.6 mg/L. Total phosphorus concentrations was lower in Guánica (< 1.25 mg/L) and Ponce Bay (< 1.47 mg/L) compared to Guayanilla Bay 3.96 mg/L and El Faro 4.08 mg/L.

Mean precipitation data seven days prior to sampling were obtained from Atmospheric Caribbean Center, University of Puerto Rico and is shown in Fig. 6. Sampling periods considered as rainy season (> 1.40 mm precipitation) included: January 2009, June 2009, October 2009, and November 2009 for Guayanilla and Ponce samples. June 2009 was used as dry season for Guánica samples (0.51 mm). December 2008, March 2009, April 2009, and May 2009 were used as dry season (< 1.40 mm). December 2008 had lower precipitation values for all sampling sites (< 0.10 mm). Guayanilla and Guánica both registered more than 6.00 mm of precipitation in January 2009.

Table 2: Sediment quality parameters in different sampling sites

Sample Site	Temperature (^{o}C)	Salinity (ppt)	pH
Guayanilla Bay			
December 2008	22.5	36.0	7.7
January 2009	21.0	36.0	7.5
March 2009	23.0	36.0	7.7
April 2009	22.5	36.0	7.6
May 2009	24.0	36.0	7.5
June 2009	30.5	36.0	7.4
October 2009	30.0	36.0	8.0
November 2009	29.0	36.0	No data
Guayanilla El Faro			
December 2008	22.0	34.0	7.9
January 2009	20.0	37.0	7.7
March 2009	24.0	39.0	8.1
April 2009	22.0	37.0	8.1
May 2009	23.0	36.0	7.8
June 2009	30.5	36.0	7.5
October 2009	32.0	34.0	8.1
November 2009	29.0	34.0	No data
Ponce Bay			
December 2008	22.0	35.0	7.9
January 2009	20.0	31.0	7.7
March 2009	22.0	36.0	7.7
April 2009	22.0	36.0	8.0
May 2009	24.0	36.0	7.7
June 2009	30.5	36.0	7.5
October 2009	31.0	35.0	8.2
November 2009	28.0	37.0	No data
Guánica			
December 2008	21.0	36.0	8.0
January 2009	20.0	35.0	7.8
March 2009	22.0	36.0	8.0
April 2009	21.0	37.0	8.0
May 2009	No data	36.0	7.9
June 2009	31.0	36.0	7.7
October 2009	29.0	37.0	8.2
November 2009	28.0	35.0	No data

Table 3: Sediment quality parameters in sampling sites during December 2008.

Study site	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	Total phosphorus (mg/L)
Guayanilla Bay	< 5	< 10.0	13.6	3.96
El Faro	< 5	10.5	42.0	4.08
Ponce Bay	< 5	< 10.0	12.9	< 1.47
Guánica	< 5	< 10.0	10.2	< 1.25

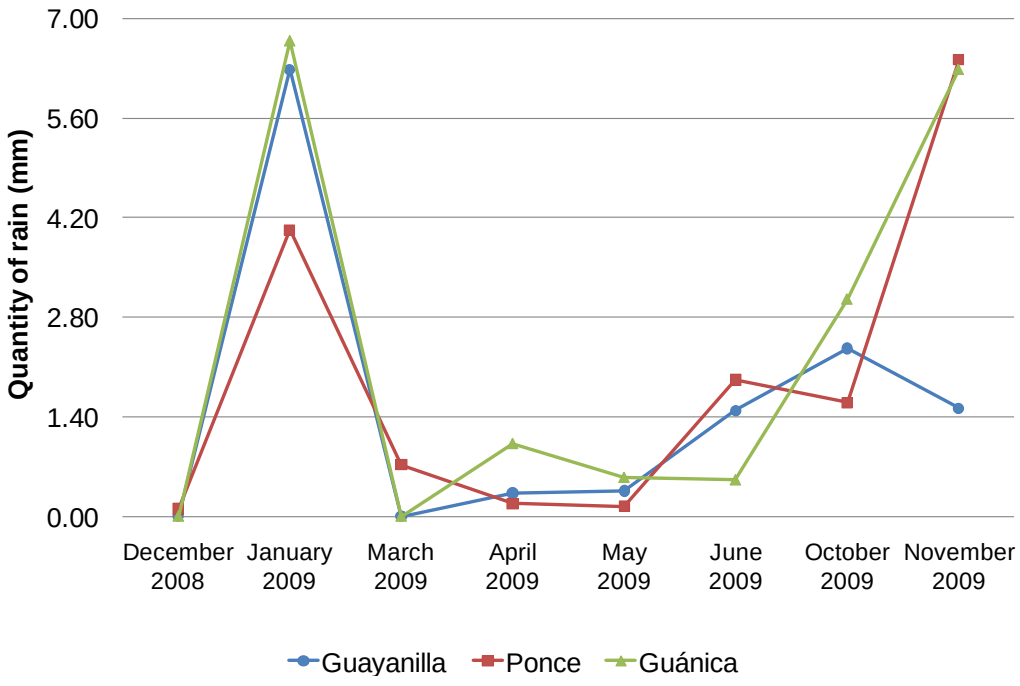


Fig. 6: Mean precipitation data 7 days prior to sampling.

Enumeration of heterotrophic bacteria

Most probable number (MPN) was used to estimate the population of heterotrophic bacteria in sediment samples during December 2008, June 2009, and November 2009 (Fig. 7). During December 2008, Guayanilla Bay and Ponce Bay had similar numbers of heterotrophic bacteria (4.60×10^5 MPN/g of sediments) while Guánica had lower MPNs (6.60×10^4 MPN/g of sediment). Higher values of estimated heterotrophic bacterial numbers were observed during June 2009 from all sampling sites. MPNs of 9.84×10^7 and 6.54×10^5 /g of sediments were found in Guayanilla Bay and in Guánica respectively. Slightly lower numbers of heterotrophic bacteria (6.56×10^6 MPN/g of sediments) were found in November 2009 in both Guayanilla and Ponce while in El Faro the numbers were 3.38×10^6 MPN/g of sediments. The numbers of heterotrophic bacteria were always lower in Guánica.

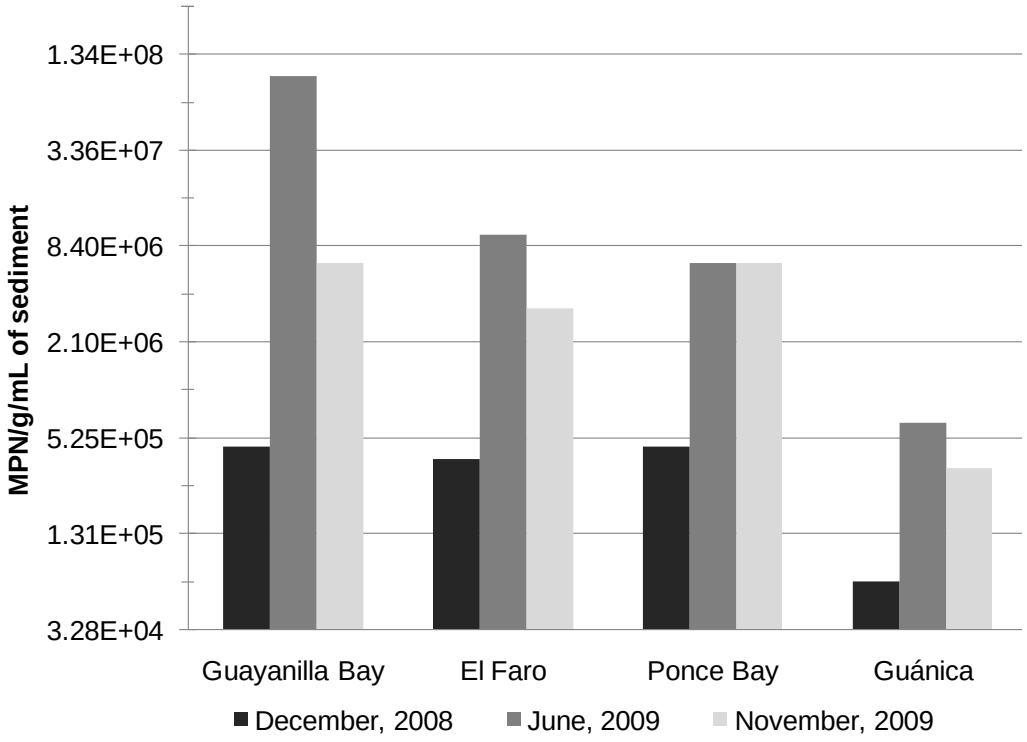


Fig. 7. Most probable number (MPN) of heterotrophic bacteria.

Optimization of procedures for the enumeration of hydrocarbon-degrading bacteria

Modifications in preparation of underlayer. The results from the modification of underlayer are shown in Fig. 8. Briefly, when the underlayer was prepared with artificial seawater without vitamins, only the Guayanilla Bay samples with 600 CFU of degrading bacteria per gram of sediment were recovered. However, phenanthrene-degrading bacteria were found in Guayanilla Bay in all underlayer treatments. No difference in the recovery of hydrocarbon-degrading bacteria was found in Ponce Bay samples when underlayer was prepared using artificial seawater or seawater (Fig. 8).

Modifications in preparation of overlayer. Compared to naphthalene higher numbers of phenanthrene-degrading bacteria were observed in Guayanilla Bay samples (2.60×10^3 CFU/g),

followed by Ponce samples with 2.36 x 10^3 CFU/g of sediments (Fig. 9). In Guánica, samples were only 90 CFU/g of phenanthrene degraders per gram of sediments. Enumeration of naphthalene-degrading bacteria was only observed in Guayanilla Bay samples. Chrysene-degrading bacteria were found in all samples with higher numbers in Guayanilla Bay 1.70 x 10^4 CFU/g followed by Ponce Bay samples 2.60 x 10^3 CFU/g of sediments (Fig. 9).

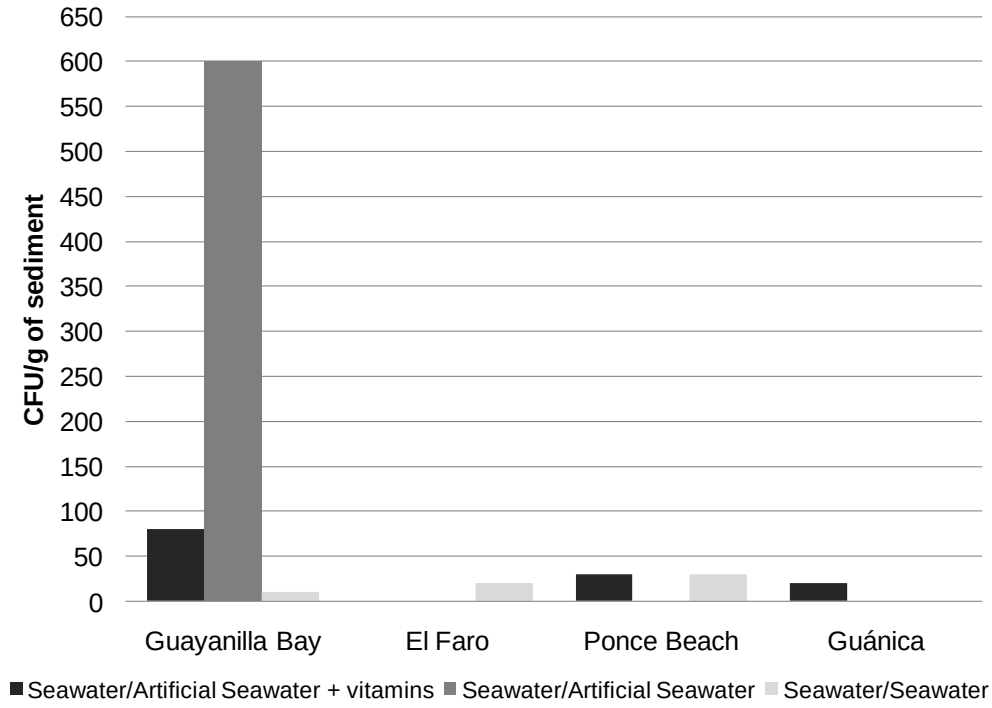


Fig. 8. Enumeration of PAHs-degrading bacteria with several underlayer modifications.

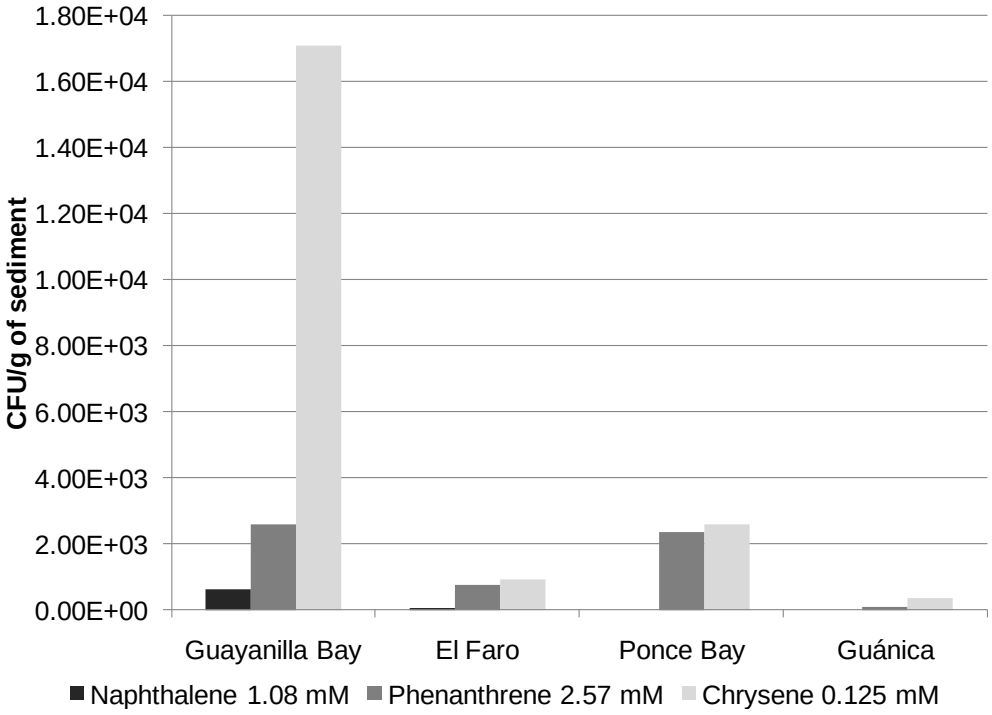


Fig. 9. Enumeration of PAHs-degrading bacteria at different sampling sites.

Concentrations of PAHs. After experimenting with different concentrations of chrysene, naphthalene, and phenanthrene, it was decided to use the same concentrations (0.25 mM) for all the PAHs substrates tested.

Enumeration of PAHs-degrading bacteria

During December 2008, lowest number of chrysene, naphthalene, and phenanthrene-degrading bacteria were found in Guánica samples (50 CFU/g of sediments). Both Guayanilla Bay and Ponce had higher number of chrysene-degrading bacteria, 2.40 $x10^3$ CFU/g and 1.56 x 10^3 CFU/g of sediments respectively. The number of chrysene-degrading bacteria in El Faro samples in December 2008 was only 260 CFU/g of sediments. The number of bacteria capable of degrading naphthalene was about 300 CFU/g of sediments in El Faro, 2.23 x 10^3 CFU/g in Guayanilla Bay, and 2 x 10^3 CFU/g in Ponce. The number of phenanthrene-degrading bacteria was similar in Guayanilla and Ponce sediments (2 x 10^3 CFU/g), but again was lower in El Faro with 240 CFU/g of sediment (Fig. 10). In January samples, 3.20 x 10^3 CFU/g naphthalene-degrading bacteria were found in El Faro, with higher numbers of 1.16 x 10^4 CFU/g in Guayanilla Bay, and only 240 CFU/g in Ponce. In Guánica, the number of chrysene, naphthalene, and phenanthrene-degrading bacteria were < 1 x 10^2 CFU/g of sediments. The numbers of phenanthrene-degrading bacteria were 2.20 x 10^2 CFU/g of sediment in El Faro and 3.10 x 10^3 CFU/g of sediment in Ponce (Fig. 11). Enumeration of hydrocarbon bacteria in March 2009 was 2.60 x 10^3 CFU/g of phenanthrene-degrading bacteria in Guayanilla Bay sediments. The number of naphthalene and chrysene-degrading bacteria was more than 1 x 10^3 CFU/g in Guayanilla Bay, but in El Faro samples the number of chrysene, naphthalene, and phenanthrene were less than 1 x 10^3 CFU/g of sediments. In Ponce sediments samples, the corresponding number of hydrocarbon-degrading bacteria was 8.50 x 10^3 CFU/g for chrysene,

5.50 x 10^3 CFU/g for naphthalene, and 9.50 x 10^3 CFU/g for phenanthrene (Fig. 12). As expected few hydrocarbon-degrading bacteria were found in Guánica (Fig. 12).

During April 2009, higher numbers of degrading bacteria of chrysene (2 x 10^4 CFU/g) and phenanthrene (1.24 x 10^4 CFU/g) were found than naphthalene (2.10 x 10^3 CFU/g) in El Faro sediments. In Guayanilla and Ponce sediment samples, phenanthrene-degrading bacteria were higher 4.10 x 10^3 CFU/g and 3.70 x 10^3 CFU/g respectively. The number of degrading bacteria of chrysene, naphthalene, and phenanthrene was negligible (< 200 CFU/g) in Guánica sediment samples (Fig. 13). In May 2009 sediment samples, the highest numbers of phenanthrene-degrading bacteria 2.60 x 10^4 CFU/g were found followed by naphthalene 2.40 x 10^4 CFU/g, and chrysene 2.30 x 10^4 CFU/g (Fig. 14). Higher numbers of phenanthrene-degrading bacteria were also found in El Faro and Ponce (Fig. 14). Guánica again had the lowest number of hydrocarbon-degrading bacteria.

Comparison of naphthalene and phenanthrene-degrading bacteria in June 2009 samples showed more naphthalene-degrading bacteria for all sampling sites. Guayanilla had the highest number of naphthalene-degrading bacteria 1.10 x 10^4 CFU/g, followed by Ponce 1.49 x 10^3 CFU/g, and El Faro 1.40 x 10^3 CFU/g (Fig. 15). Guánica sediments had lowest number of hydrocarbon-degrading bacteria with about 520 CFU/g of sediments (Fig. 15). In October 2009, highest numbers of naphthalene-degrading bacteria, 1.10 x 10^4 CFU/g were also observed in Guayanilla Bay. The number for phenanthrene and chrysene-degrading bacteria in Guayanilla sediments were 1.10 x 10^3 CFU/g and 900 CFU/g respectively (Fig. 16). Fewer hydrocarbon-degrading bacteria were found in El Faro, Guánica, and Ponce sampling sites (Fig. 16). However, in the November 2009 sampling, the pattern was changed and higher numbers of chrysene-degrading bacteria (2.50 x 10^4 CFU/g) were found in Guayanilla Bay followed by

naphthalene (1.41 x 10^4 CFU/g), and phenanthrene (5 x 10^4 CFU/g) degrading bacteria (Fig. 17). The numbers of hydrocarbon-degrading bacteria were much smaller in El Faro, Guánica, and Ponce samples (Fig. 17).

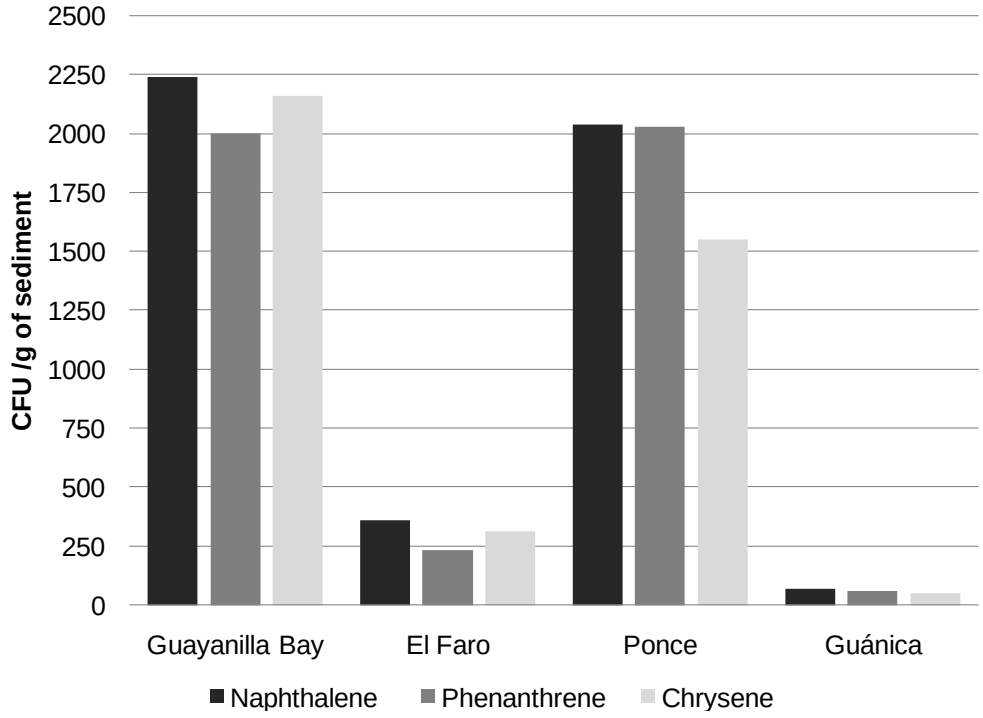


Fig. 10. Enumeration of PAHs-degrading bacteria in December 2008.

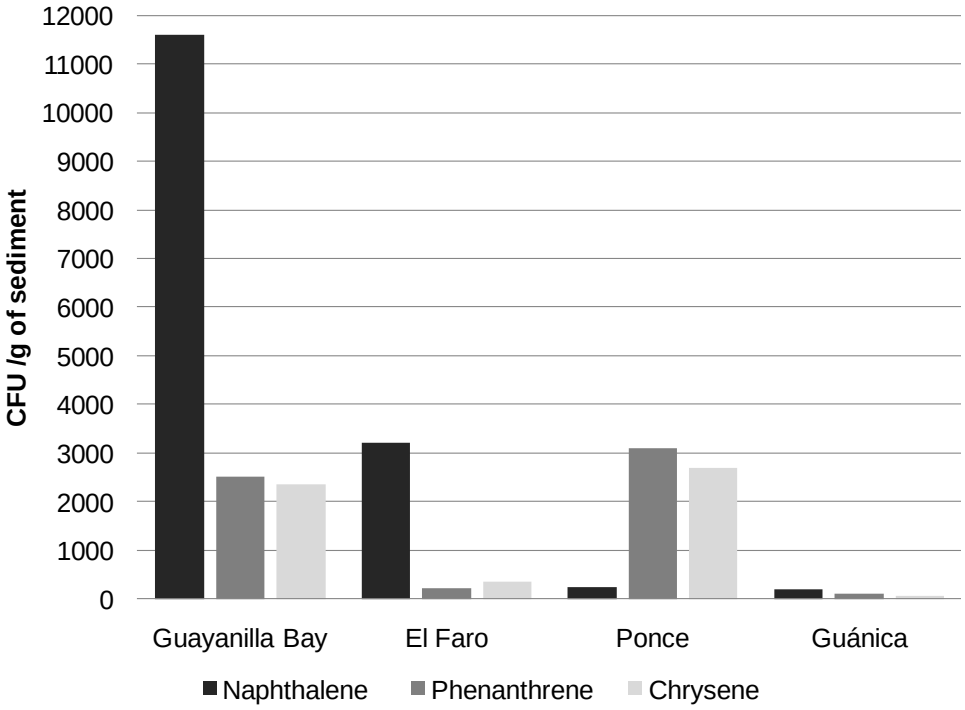


Fig. 11: Enumeration of PAHs-degrading bacteria in January 2009.

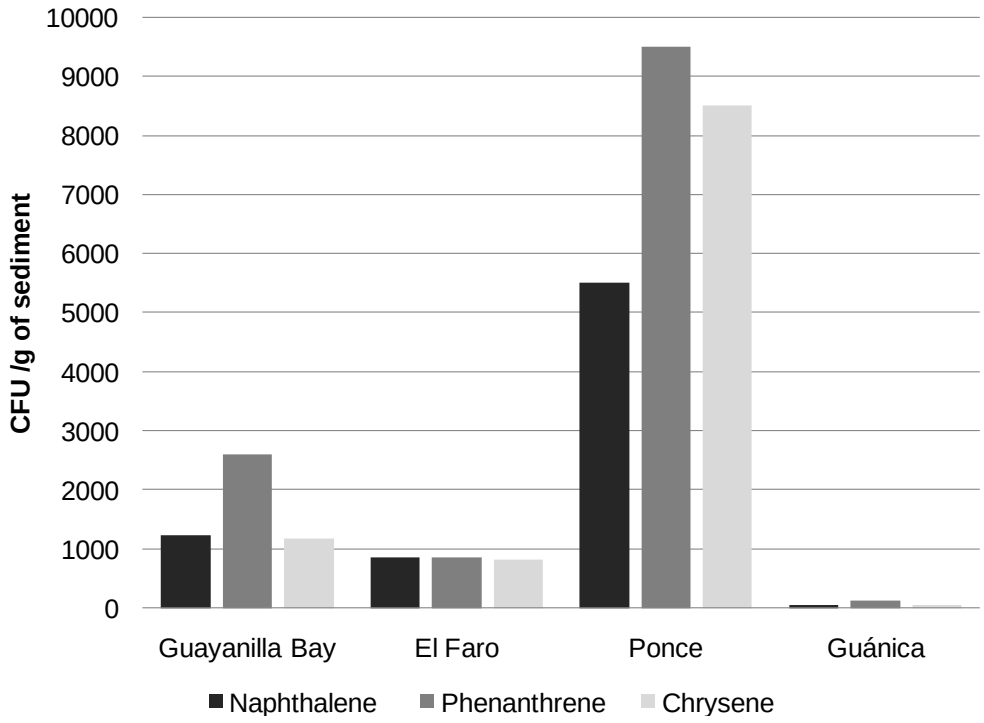


Fig. 12: Enumeration of PAHs-degrading bacteria in March 2009.

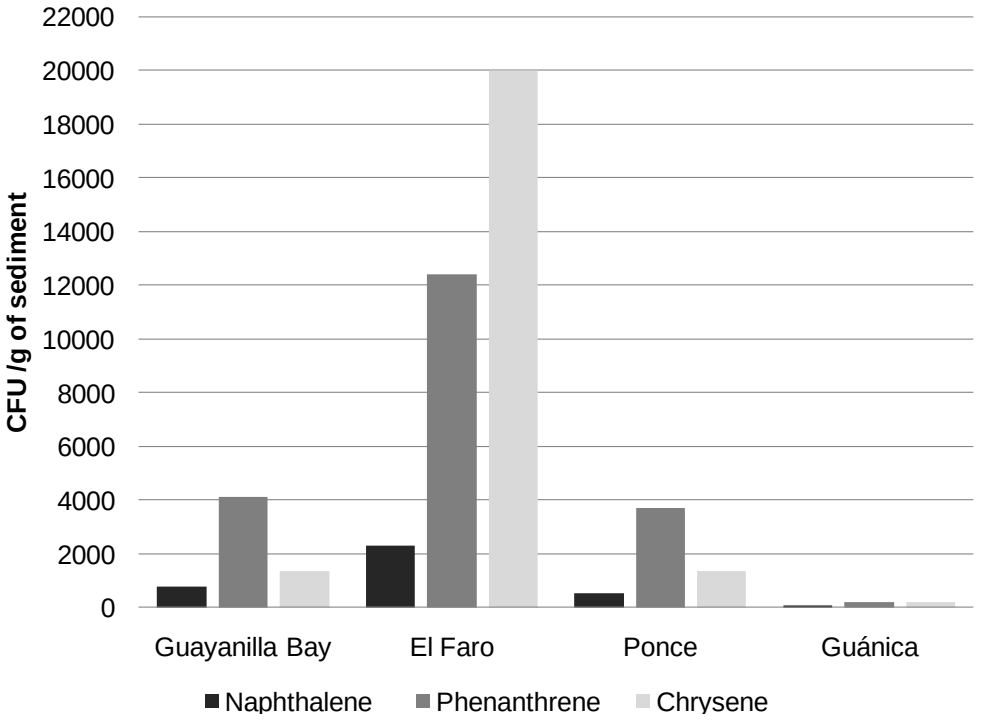


Fig. 13: Enumeration of PAHs-degrading bacteria in April 2009.

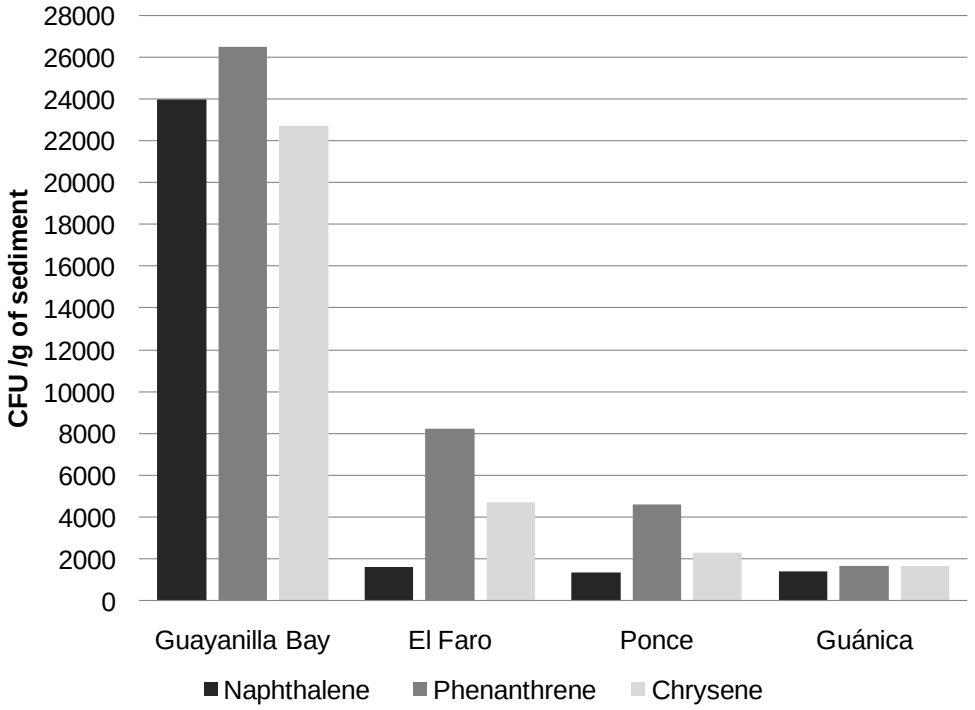


Fig. 14: Enumeration of PAHs-degrading bacteria in May 2009.

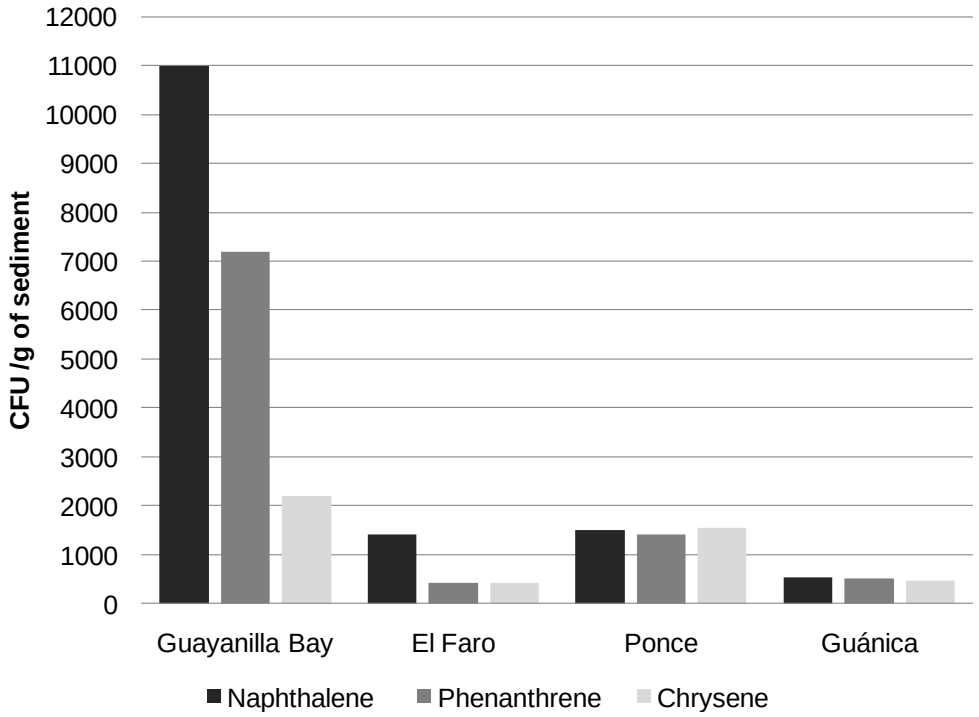


Fig. 15: Enumeration of PAHs-degrading bacteria in June 2009.

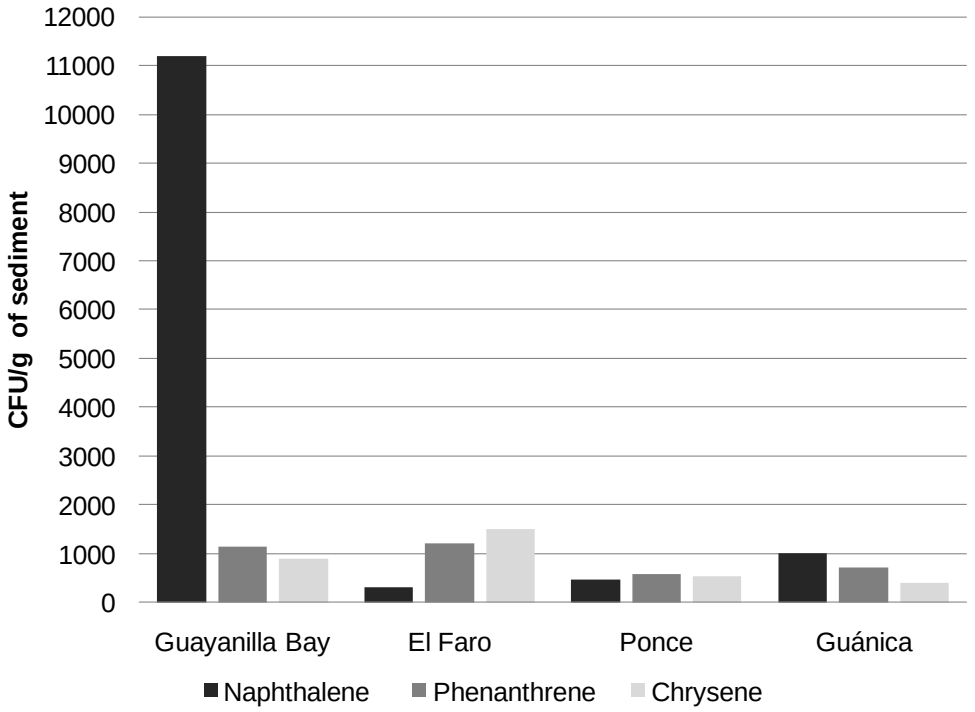


Fig. 16: Enumeration of PAHs-degrading bacteria in October 2009.

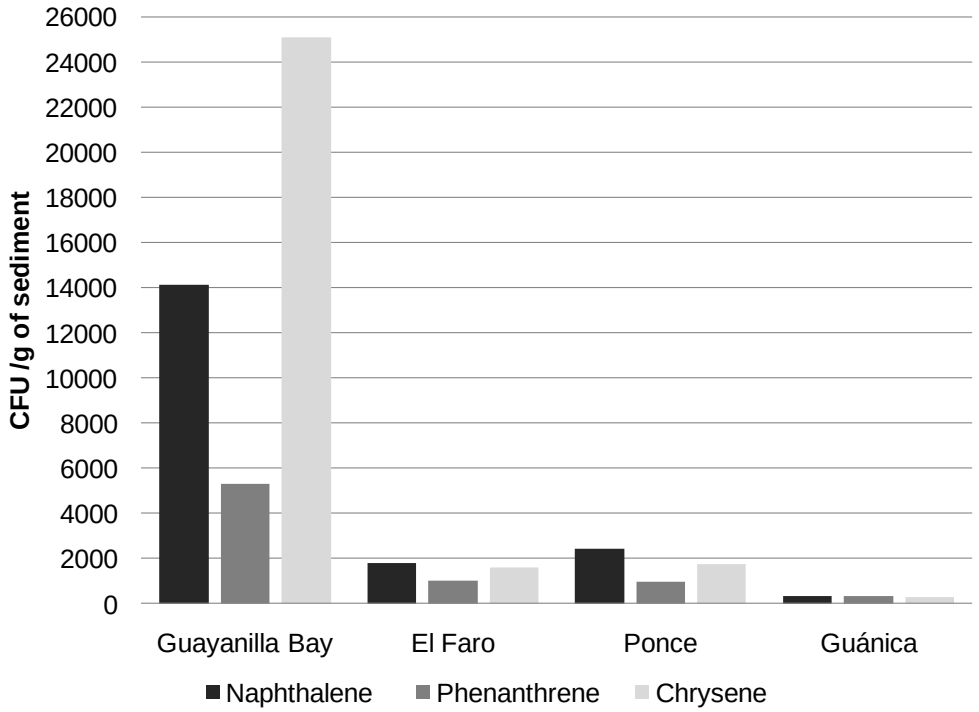


Fig. 17: Enumeration of PAHs-degrading bacteria in November 2009.

Biolog MT microplates

After 3 days of incubation at room temperature, the change of color to purple of the Biolog MT microplates was visually recorded as (+) positive growth; no color change indicated (-) negative growth. However, some of the controls also changed color. Thus, the results obtained by visual inspection of Biolog MT Micro plates were not considered satisfactory as a recommended method for color change. BIOLOG MT microplate reader and software were not available.

Isolation, purification, and identification of PAHs-degrading bacteria

Morphological characteristics

Ten phenanthrene-degrading bacteria were isolated from sampling sites (Table 4). One isolate was isolated from Guánica (1GNC) and one from El Faro (2FARO). Both strains were Gram negative, rod-shaped single pairs, with beige color, and circular morphology. One isolate from El Faro (3FARO) and another from Ponce Bay (1PORT) were isolated. Both strains were Gram negative short rods that formed circular, entire convex, and odorless colonies. After 48 hrs of incubation, these colonies changed to brown color, and noticeable odor. These isolates did not grow in TCBS and MacConkey medium.

Three other isolates (two from Ponce and one from Guánica) were Gram negative; colonies were of circular form with entire margins. Both strains grew in TCBS medium as small blue colonies. Isolates from Ponce Bay (2PORT and 3PORT) were short straight rods, while the Guánica strain (2GNC) was rod-shaped forming a chain. A Gram negative, rod shaped bacterium forming long chains with punctiform was isolated from Guayanilla Bay (1GBAY). Another strain isolated from El Faro (4FARO) was also Gram negative, rod-shaped, forming

single pairs, punctiform shaped. A Gram negative, short straight rods bacterium was isolated from El Faro samples (1FARO). This strain grew fast in TCBS medium and changed the color from green to yellow.

Table 4: Phenotypic characteristics of phenanthrene-degrading bacteria isolates.

Strain ID	Isolate from	Gram stain	Colony Morphology	Arrangement	MacConkey medium	TCBS medium
1GBAY	Guayanilla Bay	-	Puntiform, entire, beige	Chain rods	No data	No data
1FARO	El Faro	-	Circular, entire, convex, beige	Short straight rods	No data	Fast growth yellow colonies
2FARO	El Faro	-	Circular, entire, beige	Single pairs rods	No data	No data
3FARO	El Faro	-	Circular, entire, convex, beige to brown	Short straight rods	No growth	No growth
4FARO	El Faro	-	Puntiform, entire, beige	Single straight rods	No data	No data
1PORT	Ponce Bay	-	Circular, entire, convex, beige to brown	Short straight rods	No growth	No growth
2PORT	Ponce Bay	-	Circular, entire, beige to yellow	Short straight rods	Lactose +	Small blue colonies
3PORT	Ponce Bay	-	Circular, entire, beige to yellow	Short straight rods	Lactose +	Small blue colonies
1GNC	Guánica	-	Circular, entire convex, beige	Single pairs rods	Lactose +	No data
2GNC	Guánica	-	Circular, entire, beige to yellow	Chain rods	Lactose +	Small blue colonies

Substrate utilization patterns of phenanthrene-degrading bacteria strains

Substrate utilization patterns for α-methyl-glucose, arabinose, arginine, manitol, ribose, and sorbose were investigated by phenanthrene-degrading bacteria isolates (Table 5). The bacteria isolated from El Faro (4FARO), Guánica (1GNC), Guayanilla Bay (1GBAY), and Ponce (2PORT) did not utilize any of the substrates tested. Strains isolated from Ponce Bay (3PORT) and Guánica (2GNC) used manitol, indicated by positive growth. The El Faro strain (1FARO) used manitol, sorbose, and sucrose. Another strain from El Faro (2FARO) used manitol, ribose, and sucrose as substrates. Ribose was only used by the El Faro strain (3FARO), while the isolate from Ponce Bay (1PORT) only used sucrose. None of the isolated strains used α-methyl-glucose, arabinose, and arginine as substrate (Table 5).

Table 5: Substrate utilization patterns of phenanthrene-degrading bacteria isolates.

Strain ID	Arginine	Arabinose	Manitol	Ribose	Sorbose	Sucrose	α-methyl-glucose
1GBAY	-	-	-	-	-	-	-
1FARO	-	-	+	-	+	+	-
2FARO	-	-	+	+	-	+	-
3FARO	-	-	-	+	-	-	-
4FARO	-	-	-	-	-	-	-
1PORT	-	-	-	-	-	+	-
2PORT	-	-	-	-	-	-	-
3PORT	-	-	+	-	-	-	-
1GNC	-	-	-	-	-	-	-
2GNC	-	-	+	-	-	-	-

Identification of bacterial strains by molecular techniques

DNA fingerprinting of bacterial isolates and phylogenetic analysis

PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega). Product concentration was determined using 1% agarose gel with markers *Hind* III (Fig. 18). Analysis of partial 16S rDNA gene sequences of phenanthrene-degrading bacteria isolates is shown in Table 6. The sequence analysis was performed using the RDP database with NCBI related strains. Eight sequences, which were the majority of the sequences had molecular size fragments ranging between 678 pb to 762 pb. However, 1GBAY had a molecular size fragment of 821pb while 1FARO had a fragment length of 542 pb. Similarity coefficients ranged from 0.893 to 0.957, except for 1FARO isolate with 0.742.

Three phylogenetic trees were constructed with the obtained sequences (Nevada Genomics). Neighbor-Joining method and *p*-distance mode were used to generate the phylogenetic trees. Figure 19 shows eight of the sequences with molecular size fragment ranging from 678 pb to 762 pb. Figures 20 and 21 show the phylogenetic tree of isolates 1GBAY and 1FARO, respectively. Numbers on the branches indicate percent bootstrap values with more than 50% bootstrap support. 1GNC and 2FARO strains have homology with *Alteromonas* sp. On the other hand, strains isolated from Ponce beach (2PORT and 3PORT) and 2GNC are related with *Vibrio agarivorans*. Correlation with *Microbulbifer elongatus* was observed for 3FARO and 1PORT. An isolate, 4FARO have 100% of homology with a marine bacterium EU513001. All the strains were phylogenetically related to the bacterial division γ-*Proteobacteria*. The out-group used to root the tree was *Bacillus cereus* HQ400609.1. This Gram-positive bacterium was selected because *Bacillus cereus* is phylogenetically outside the group of species specifically being studied.

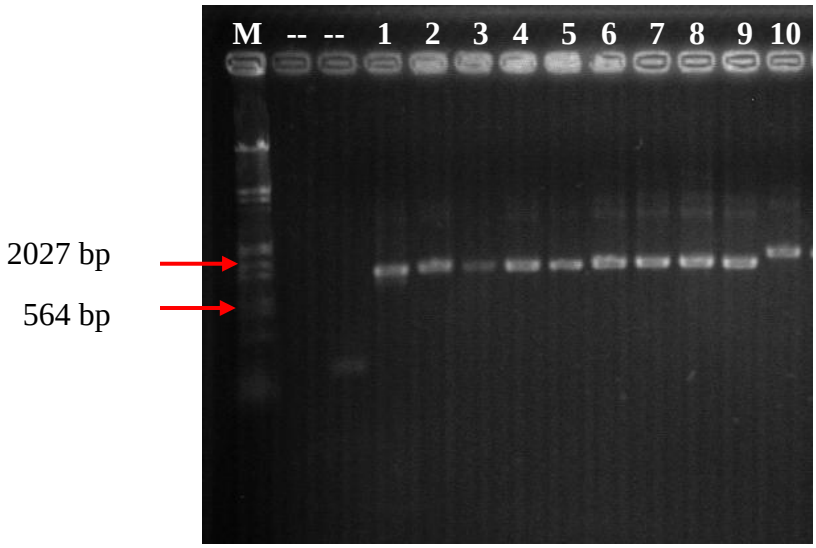


Figure 18: PCR products from phenanthrene-degrading isolates.

Table 6: Analysis of partial 16S rDNA gene sequences isolated from phenanthrene-degrading bacteria strains.

Strain ID	Fragment length (bp)	[1]RDP related strain	[2]Sab
1GBAY	821	*Vibrio furnissii* (T)	0.914
1FARO	542	*Vibrio furnissii* (T)	0.742
2FARO	688	*Alteromomas* sp.	0.919
3FARO	749	*Microbulbifer elongatus* (T)	0.957
4FARO	740	marine bacterium EU513001	0.893
1PORT	736	*Microbulbifer elongatus* (T)	0.909
2PORT	678	*Vibrio agarivorans*(T)	0.925
3PORT	695	*Vibrio agarivorans*(T)	0.906
1GNC	733	*Alteromomas* sp.	0.948
2GNC	762	*Vibrio agarivorans*(T)	0.936

[1]Ribosomal Database Project
[2]Similarity coefficient

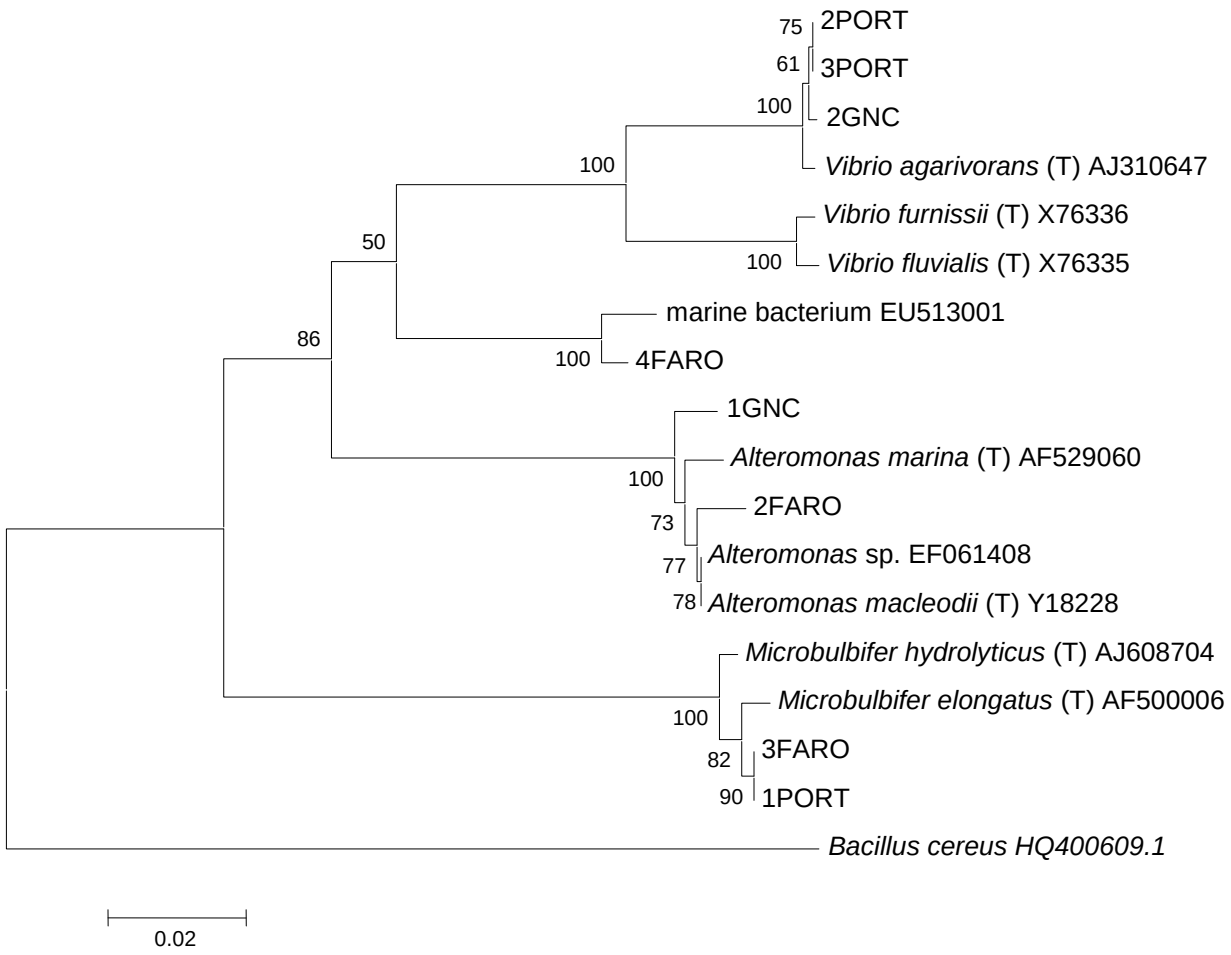


Figure 19: Phylogenetic tree constructed using the neighbor-joining method and *p*-distance mode for eight phenanthrene-degrading bacteria isolates. The scale bar represents 0.02 Jukes-Cantor substitutions per nucleotide.

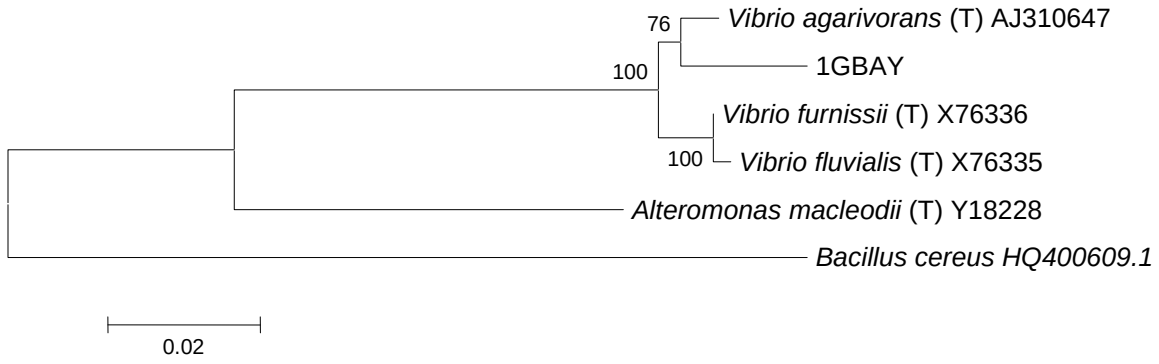


Figure 20: Phylogenetic tree constructed using the neighbor-joining method and *p*-distance mode for 1GBAY isolate. The scale bar represents 0.02 Jukes-Cantor substitutions per nucleotide.

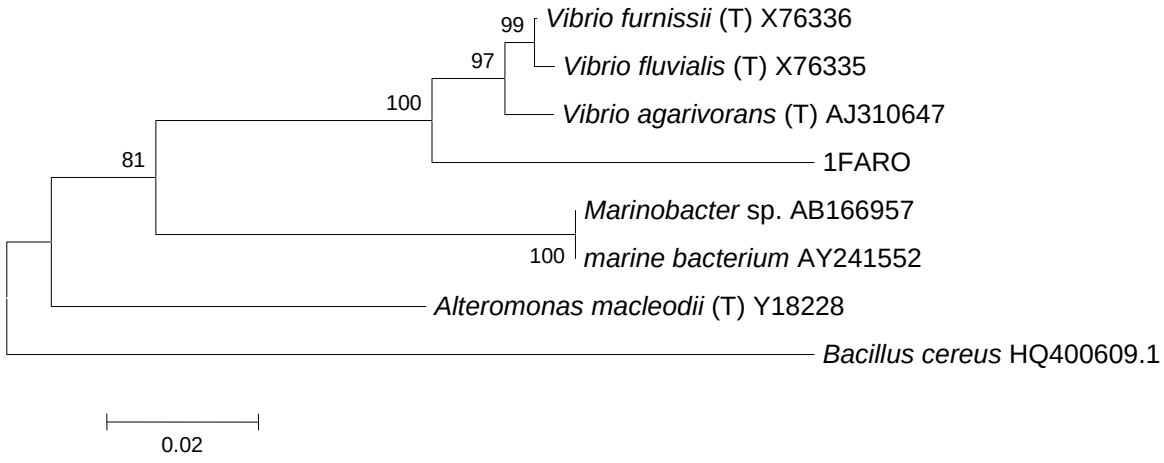


Figure 21: Phylogenetic tree constructed using the neighbor-joining method and *p*-distance mode for 1FARO isolate. The scale bar represents 0.02 Jukes-Cantor substitutions per nucleotide.

Discussion

Sediment quality parameters

Overall no significant differences were found in sediment quality parameters (pH, salinity, and temperature). However, notable difference in nitrate concentration was observed in El Faro sample. Previous studies in Guayanilla Bay demonstrated high concentration of nitrate in this area (Lair *et al*., 1971). High values in nitrate and phosphorus concentrations may be due to mangrove swamps near the coastline of El Faro. According to Fernandes *et al.*, (2010), sediments in mangrove habitats could act as a sink of nitrate if organic carbon input is not favorable to the denitrifying process. The shallow waters in the intertidal zone found in El Faro may contribute to sequestering these nutrients in the marine sediments. Other factors that can contribute with nutrient input are the Guayanilla River and the discharge of effluents from a nearby sewage treatment plant.

Heterotrophic bacteria

Higher numbers of heterotrophic bacteria were observed for all sampling sites during June 2009 and November 2009. Rainfall events may contribute to the input of organic matter and nutrients to coastal water by mixing bottom sediments that provide surface area for microorganism growth. Higher number of heterotrophic bacteria in Guayanilla Bay may be due to the influence of Macaná River. Low organic carbon accumulation and absence of river input in Guánica sediments may contribute to low heterotrophic bacterial numbers in Guánica sediments.

Modifications to optimize enumeration of PAHs-degrading bacteria

No growth of hydrocarbon bacteria was observed in El Faro samples when the underlayer was prepared with artificial seawater. This may be due to the absence of essential nutrients or growth factors lacking in artificial seawater. According to Poeton (1999), the presence of growth factors in sediment enhance the rates of degradation by bacteria attached to sediment. Guayanilla sediments may have growth factors derived from the Guayanilla River. The higher numbers of hydrocarbon-degrading bacteria in Guayanilla Bay were present when the underlayer was prepared with artificial seawater alone compared to artificial seawater amended with vitamins, indicating that the presence of vitamins is not essential for growth of these bacteria in Guayanilla Bay sediments.

Naphthalene-degrading bacteria at 1.08 mM naphthalene concentration were only found in the Guayanilla samples. This suggests two possibilities, that the concentration of naphthalene in the Ponce and Guánica sediments was low, so that there were few naphthalene-degrading bacteria in the sediments. The second possibility is that there were high levels of naphthalene at these sites, so that the toxicity of this compound was sufficiently high to inhibited the growth of the naphthalene-degrading bacteria. Exposure of Guayanilla Bay sediments to PAHs over a long period of time may have contributed to observed enumeration of naphthalene-degrading bacteria.

Enumeration PAHs- degrading bacteria

Slightly higher values of naphthalene-degrading bacteria were observed during the rainy season when compared with phenanthrene-degrading bacteria numbers (Fig. 22). The reason for the increase of naphthalene degraders may be due to the input of nutrients through the river effluents and mixing of bottom sediments that help in the bioavailability of nutrients, and can lead to increased numbers of degraders. Also, protozoan may contribute to increased surface

area to hydrocarbon-degrading bacteria and provide growth factors through excretion (Tso and Taghon, 2006). Another reason to explain the increase in naphthalene-degrading bacteria numbers may be due to reduction of naphthalene toxicity. During January 2009 unusually low salinity may be responsible for lower numbers of naphthalene-degrading bacteria in Ponce samples. Salinity can affect the solubility, and hence the toxicity of hydrocarbon in marine sediments. According to Shiaris (1989), negative correlation with salinity was observed in sediment samples in undisturbed sampling sites, and might explain a possible differential effect of salinity in the naphthalene and phenanthrene degradation pathways. Decrease in salinity can adversely affect obligate marine hydrocarbon degrading bacteria (Shiaris, 1989).

Compared to June and November sampling, in October 2009 sampling the temperature was high (31-32^{o}C) in El Faro and Ponce sediments. In tropical environment the temperature fluctuations beyond optimum temperature may drastically affect the growth of microorganisms. Previous work (Zaidi and Imam, 1999) in Guayanilla Bay water has shown that the growth of bacterium capable of degrading phenanthrene is adversely affected at temperature of less than 15^{o}C and higher than 30^{o}C. Besides temperatures other factors may affect the numbers of hydrocarbon-degrading bacteria, for example availability and seasonal variation of simple carbohydrates in organically rich sediments may lead to diminished numbers of PAH degraders by simple competition among them (Shiaris, 1989).

Higher numbers of phenanthrene-degrading bacteria were obtained during the dry season compared to naphthalene-degrading bacteria (Fig. 23). According to Poeton *et al*. (1998), more bacteria were found attached to sediment particle surfaces when phenanthrene was adsorbed, contributing to improved degradation rate near sediment particles. Other possible explanations for increased phenanthrene degradation during the dry season may be due to exclusion of

naphthalene-degrading bacteria, absence of the requirements necessary for growth in naphthalene enriched areas, and the presence of selective group of phenanthrene degraders. According to Aitken *et al*. (1998), some isolates of phenanthrene-degrading bacteria cannot oxidize naphthalene because the requirements for growth on naphthalene exceed the requirements of phenanthrene.

During March 2009, higher numbers of hydrocarbon degrading bacteria were observed in Ponce samples may have been due to dredging activities to the expansion of the Las Américas port that can contribute to mixing of sediments. This activity can lead to increases in the numbers of attached bacteria to sediments and contribute to nutrient bioavailability. Statistically significant higher numbers of PAHs-degrading bacteria were found in Guayanilla Bay during May 2009, may be due to an oil spill reported by the National Response Center at this time. Introduction of oil to Guayanilla Bay waters can contribute to this increase in hydrocarbon-degrading bacteria. Bacterial communities that are exposed to hydrocarbon contamination over long periods of time develop increased capabilities for biodegradation of PAHs than bacterial communities in pristine environments (Rowland *et.al*., 2000). Guayanilla Bay had the highest number of hydrocarbon-degrading bacteria. Guánica, due to lower exposure to PAHs, was used as control study site and as expected had low enumeration of hydrocarbon-degrading bacteria. According to Atlas (1981), the proportion of hydrocarbon-degrading bacteria can increase more than 10% of the total bacterial population when the environment is contaminated with petroleum.

Other factors that may affect enumeration of hydrocarbon-degrading bacteria are related to aeration, concentration of total PAHs in sediments, and grain size. Inspite of high input of nutrients into the El Faro and its historical exposure to PAHs, this site has coarse sediments compared to Guayanilla Bay sediments (Pait, 2008). Sediment samples in southern coastal of

Puerto Rico showed higher relationships between grain size (silt/clay fraction) and the concentration of total PAHs. Visual observations of the sampling sites indicated two points that may contribute to the higher enumeration of PAHs in the Guayanilla Bay samples: silt/clay fractions observed in the sediments and higher total organic carbon. A study by Santas and Santas (2000) showed that moderate wave action accelerates hydrocarbon biodegradation in sediments of the intertidal zone. However, Guánica sediments samples are mainly formed by calcareous material; the high energy (wave action) observed in this sampling site may not affect the enumeration of hydrocarbon-degrading bacteria. Also, degradation of phenanthrene in soil samples was significantly increased when samples were treated under aeration (Lorraine *et al.*, 2009).

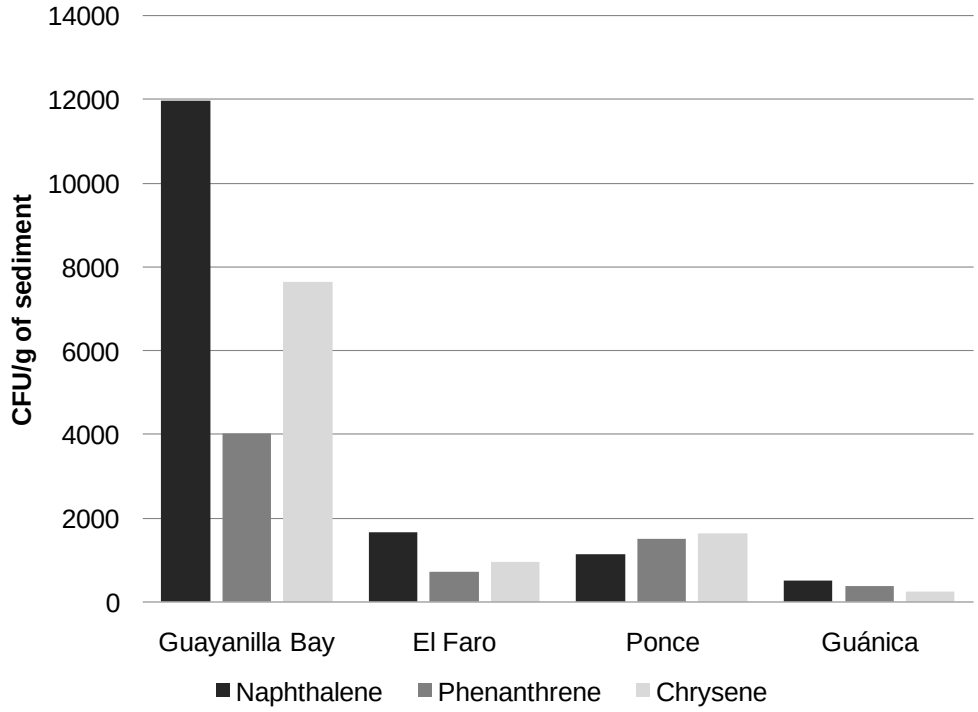


Fig. 22: Comparison between naphthalene, phenanthrene, and chrysene-degrading bacteria during the rainy season for each sampling site.

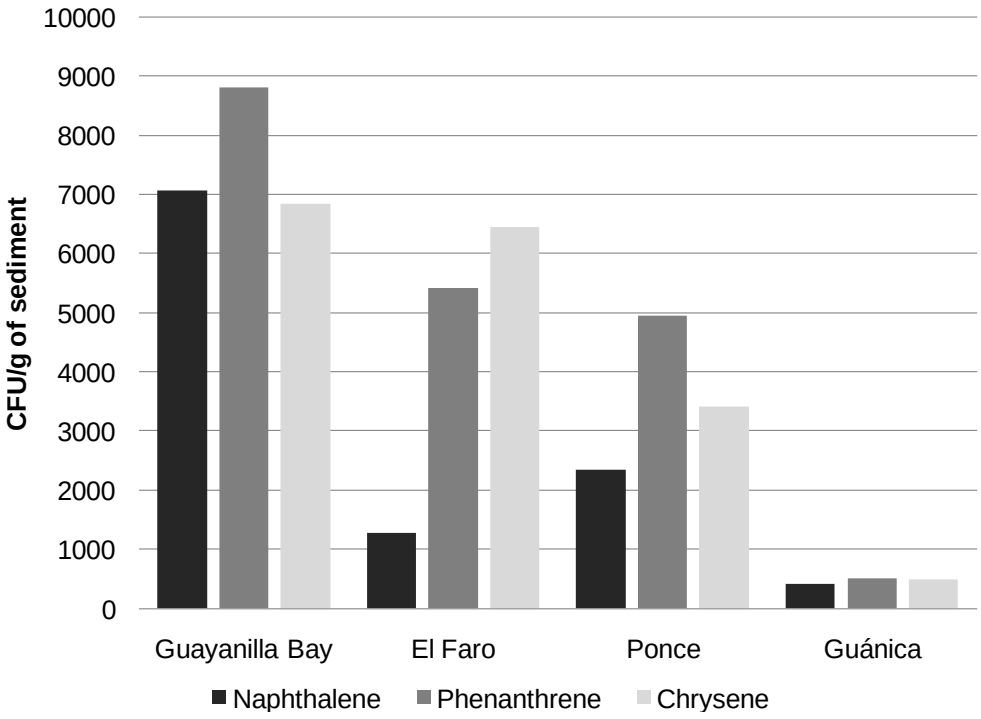


Fig. 23: Comparison between naphthalene, phenanthrene, and chrysene-degrading bacteria during the dry season for each sampling site.

Substrate utilization patterns

Pattern of utilization of substrate was different for bacteria isolated from El Faro than bacteria isolated from Guánica, Guayanilla Bay, and Ponce Bay. That may be due to the input of organic matter from land. The utilization of substrate cannot always be used as the key to differentiate among different bacterial groups. For example, even though 2PORT and 3PORT use different substrates (manitol substrate utilization by 3PORT) they still belong to the same genera, *Vibrio* sp. Differences in substrate patterns are highly influenced by the substrate availability in the environment, seasonal fluctuations and bacterial adaptability to use a particular substrate.

Phylogenetic Analysis

All isolated bacteria were Gram negative. Usually, in hydrocarbon contaminated environments, Gram negative bacteria dominate the system (MacNaughton *et al.,* 1999). Phylogenetic trees revealed that the bacterial isolates belong to the gamma *Proteobacteria* group. The *Proteobacteria* is composed of representative strains which have been well-documented as petroleum hydrocarbon degrading species (Dojka *et. al*., 1998). The three genera represented were *Alteromonas, Microbulbifer,* and *Vibrio* spp. *Alteromonas* sp. was previously isolated from Guayanilla Bay waters as phenanthrene-degrading bacteria (Zaidi *et al.,* 2003). Several authors isolated *Vibrio* sp. as phenanthrene degraders in coastal sediments (Berardesco *et al.,* 1998 and West *et al.,* 1984). Representative of this genus were isolated from all sampling sites. On the other hand, a *Microbulbifer* sp. was previously isolated from mangrove sediments in Brazil (Brito *et al.,* 2006). The ecological role in the aromatic hydrocarbon degradation by *Microbulbifer* sp. in these ecosystems is considered important. Phylogenetic analysis of the isolates indicated 4FARO as forming a new branch with marine bacterium EU513001 and may

belong to a new genus. On the other hand, 1FARO does not show homology with any previously described genus. The fragment length (540 pb) generated for 1FARO is relatively short to compare accurately with the database sequences.

Conclusions

- Hydrocarbon degradation capabilities to use chrysene, naphthalene, and phenanthrene as carbon source were observed in El Faro, Guánica, Guayanilla Bay, and Ponce Bay sediments.
- Enumeration of heterotrophic bacteria was higher in rainy periods (June and November samples). Rain events may have introduced nutrients and substrates to coastal environments that favored bacterial growth.
- Enumeration of hydrocarbon-degrading bacteria was higher for Guayanilla Bay and lower for Guánica.
- Enumeration of hydrocarbon-degrading bacteria was influenced by probably resuspension of the sediments and availability of nutrients caused by rain and by river effluents.
- Higher enumeration of hydrocarbon-degrading bacteria in Guayanilla Bay sediments may be due to the historical contamination because of its location in and vicinity of a petrochemical complex.
- Highest enumeration of PAHs-degrading bacteria in May 2009 may be due to reported an oil spill during the sampling period.
- Enumeration of naphthalene-degrading bacteria was observed in rainy periods while phenanthrene-degrading bacteria predominated in dry periods.
- PAHs-degrading bacteria isolates are related to *Alteromonas* sp., *Microbulbifer* sp., and *Vibrio* sp. These isolates have previously been isolated from other hydrocarbon contaminated sites.

Recommendations

According to the findings of this study, the following recommendations are proposed:

- Sediment quality parameters such as determination of ammonia, nitrite, nitrate and phosphorus, grain size, and total organic carbon for each sample period are necessary to evaluate possible abiotic factors that can affect the fate and transport of PAHs.
- Study of bioavailability of nutrients and PAHs according to the characteristics of sediments.
- Study different methods of dispersion of marine sediments in the laboratory to isolate PAHs degrading bacteria.
- Determination of minimum inhibitory concentration of different substrates in PAHs-degrading bacteria.
- Study the use of different filters to maximize the recovery of bacteria from the sediment samples.
- Use culture independent techniques to determine diversity of PAHs-degrading hydrocarbon bacteria in sediment samples.

References

Aitken, M. D., W. T. Stringfellow, R. D. Nagel, C. Kazunga and Shu-Hwa Chen (1998). Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. ***Canadian Journal of Microbiology***. 44(8):743-752.

Atlas, R. M. (1995). Petroleum biodegradation and oil spill bioremediation. ***Marine Pollution Bulletin***. 31(4-12):178-182.

Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. ***Microbiological Reviews***. 45(1):180-209.

Amellal, N., J. M. Portal and J. Berthelin (2001). Effect of soil structure on bioavailability of polycyclic aromatic hydrocarbons within aggregates of a contaminated soil. ***Applied Geochemistry***, 16(14):1611-1619.

Arbabi, M., S. Nasseri, A. R. Mesdaghinia, S. Rezaie, K. Naddafi, G. H. Omrani and M. Yunesian (2004). Survey on physical, chemical and microbiological characteristics of PAH-contaminated soils in Iran. ***Iranian Journal Environmental Health Science Engineering***. 1(1):26-33.

Berardesco, G., S. Dyhrman, E. Gallagher and M. P. Shiaris (1998). Spatial and temporal variation of phenanthrene-degrading bacteria in intertidal sediments. ***Applied Environmental Microbiology***. 64(7):2560-2565.

Bogardt, A. H. and B. B. Hemmingsen (1992). Enumeration of phenanthrene-degrading bacteria by an overlayer technique and its use in evaluation of petroleum-contaminated sites. ***Applied Environmental Microbiology*** 58(8):2579-2582.

Brito, E. M., R. Guyoneaud, M. Goni-Urriza, A. Ranchou-Peyruse, A. Verbaere, M. A. Crapez, J. C. Wasserman and R. Duran (2006) Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay, Brazil. ***Research in Microbiology*** **157(8)**: 752–762

Cernilia, C. E., (1992). Biodegradation of polycyclic aromatic hydrocarbons. ***Biodegradation***. 3:351-368.

Chartock, M. A. (1980). ***Hydrological model of Guayanilla Bay, Puerto Rico***. Research Project CEERM-64. Center for Energy and Environmental Research. University of Puerto Rico. U.S. Department of Energy. pp. 1-16.

Chung, W. K. and G. M. King (2001). Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of *Lutibacterium anuloederans* gen. nov., sp. nov., and *Cycloclasticus spirillensus* sp. nov. ***Applied and Environmental Microbiology***. 67(12):5585-5592.

Dojka, M. A., P. Hugenholtz, S. K. Haack and N. R. Pace (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. ***Applied and Environmental Microbiology***. 64(10):3869-3877.

Eganhouse, R. P. and I. R. Kaplan (1982). Extractable organic matter in municipal wastewaters. 1. Petroleum hydrocarbons: Temporal variations and mass emission rates to the ocean. ***Environmental Science and Technology***. 16(3):180-186.

Environmental Quality Board (1972). ***Report on oceanographic baseline data for near shore areas along the coast of Puerto Rico***. Environmental Quality Board of the Department of Publics Works of Puerto Rico. 95 pp.

Fernandes, S. O. and P. A. Loka (2010). Nitrate levels modulate denitrification activity in tropical mangrove sediments (Goa, India). ***Environmental Monitoring and Assessment***. DOI 10.1007/s10661-010-1375-x

Fuentes**,** F. and A. A. Massol-Deyá (1996). Ecología de microorganismos. Manual de laboratorio. Editorial de la Universidad de Puerto Rico. Mayagüez, Puerto Rico. pp. 195- 201.

García, J. R. (2003). Biological characterization and mapping of marine habitats in Ponce Bay, Puerto de Las Americas. ***Final Report submitted to the PR Infrastructure Financing Authority (PRIFA)***. 45 pp.

Gibson, D. T. (1971). The microbial oxidation of aromatic hydrocarbons. ***Critical Reviews in Microbiology***. 1(2):199-223.

Goldman, G.C. (1978). ***Physical Oceanography of Guayanilla Bay***. Project PRNC-179. Center for Energy and Environmental Research. University of Puerto Rico. U.S. Department of Energy. pp.1-15.

Gonçalves, R., M Sholze, A. M. Ferreira, M. Martins and A. D. Correia (2008). The joint effect of polycyclic aromatic hydrocarbons on fish behavior. ***Environmental Research*** 108(2):205-213.

Harayama, S., Y. Kasai and H. Akihiro (2004) Microbial communities in oil-contaminated seawater. ***Current Opinion in Biotechnology*** 15:205-214.

Head, I. M. and R. PJ. Swannell (1999) Bioremediation of petroleum hydrocarbon contaminants in marine habitats. ***Current Opinion in Biotechnology***. 10:234-239.

Hinga, K. R. (2003). Degradation rates of low molecular weight PAH correlate with sediment TOC in marine subtidal sediments. ***Marine Pollution Bulletin***. 46(4):466-474.

Incardona, J. P., T. K. Collier and N. L. Scholz (2004). Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. ***Toxicology Applied Pharmacology***. 196 (2):191-205

Jong-Su, S., Y. S. Keum. and Q. X. Li (2009). Bacterial degradation of aromatic compounds. ***International Journal of Environmental Research and Public Health.*** 6:278-309.

Lair, M. D., R. G. Rogers and M. R. Weldon (1971). ***Environmental effects of petrochemical waste discharges on Tallaboa and Guayanilla Bays, Puerto Rico.*** Technical Study TS03-71-208-02. EPA. Environmental Protection Agency Region IV. Surveillance and Analysis Division. Athens. Georgia. 47 pp.

Lorraine, M. M., R. J. Grant, N. J. W. Clipson, and E. M. Doyle (2009). Bacterial community dynamics during bioremediation of phenanthrene and fluoranthene-amended soil. ***International Biodeterioration & Biodegradation***. 63(1):52-56.

MacNaughton, S. J., J. R. Stephen, A. D. Venosa, G. A. Davis, Y. Chang and D. C. White (1999). Microbial population changes during bioremediation of an experimental oil spill. ***Applied Environmental Microbiology***. 65(8):3566-3574.

Manero, A. and A. R. Blanch (1999). Identification of *Enterococcus* sp. with a biochemical key. ***Applied and Environmental Microbiology*** 65(10):4425-4430.

McClendon, J. F., C. C. Gault and S. Mulholland (1917). The hydrogen-ion concentration, CO_2-tension, and CO_2 content of sea water. In: Sverdrup, H. U., M. W. Johnson and R. H. Fleming, (eds), ***The Oceans***, Prentice-Hall, Inc. pp.186.

Miriam, Nwanna I. E., G. O. George and I. M. Olusoji (2006) Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. ***African Journal of Biotechnology*** 5(10):823-828.

Moody, J. D., J. P. Freeman, D. R. Doerge and C. E. Cerniglia. (2001). Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. ***Applied and Environmental Microbiology***. 67(4):1476-1483.

Mrozik, A., Z. Piotrowska-Seget and S. Łabużek (2003). Bacterial degradation and Bioremediation of polycyclic aromatic hydrocarbons. ***Polish Journal of Environmental Studies*** 12(1):15-25.

Neff, J. M., S. A. Stout and D. G. Gunster (2005). Ecological risk assessment of polycyclic aromatic hydrocarbons in sediments: Identifying sources and ecological hazard. ***Integrated Environmental Assessment and Management***. 1(1):22-33.

Nikolaou, A., M. Kostopoulou, G. Lofrano and S. Meric (2009). Determination of PAHs in marine sediments: analytical methods and environmental concerns. ***Global NEST Journal***. 11(4):391-405.

Pait, A. S., D. R. Whitall, C. F. G. Jeffrey, C. Caldow, A. L. Mason, G. G. Lauenstein and J. D. Christensen (2008). Chemical contamination in southwest Puerto Rico: An assessment of organic contaminants in nearshore sediments. ***Marine Pollution Bulletin***. 56(3):580-587.

Poeton, T. S., H. D. Stensel and S. E. Strand (1999). Biodegradation of Polyaromatic hydrocarbons by marine bacteria: effect of solid phase on degradation kinetics. ***Water Research***. 33(3):868-880.

Rigau, J. J. and R. H. Sardina (1980). Health Hazards Related to the Disposal of Toxicants in the Puerto Rican Environment: A case for the research involvement of the academic community and the generation of the basic data for broad based action. ***Report by the Energy and Environment Dynamics***, Inc., Río Piedras, Puerto Rico. 130 pp. [unpublished report for the PR Cancer Center, Medical Sciences Campus, UPR Río Piedras.]

Rodríguez, E. M., E. X. Pérez, C. H. Schadt, J. Zhou and A. A. Massol-Deyá (2006). Microbial diversity and bioremediation of hydrocarbon-contaminated aquifer (Vega Baja, Puerto Rico). ***International Journal of Environmental Research and Public Health***. 3(3):292-300.

Rodríguez, N. J., A. Massol., S. H. Imam and B. R. Zaidi (2007). Microbial utilization of toxic chemicals in surface waters of Guayanilla Bay, Puerto Rico: Impact of seasonal variation. ***Caribbean Journal of Science***. 43(2):172-180.

Rowland, A. P., D. K. Lindley, G. H. Hall, M. J. Rossall, D. R. Wilson, D. G. Benham, A. F. Harrison, R. E. Daniels (2000). Effects of beach sand properties, temperature and rainfall on the degradation rates of oil buried oil/beach sand mixtures. ***Environmental Pollution***. 109:109-118.

Saano A., Tas E., Piippola S., Lindstrom K. and J. V. Elsas (1995). ***Nucleic acids in the environment: methods and applications***. Trevors, J.T. and J. V. Elsas, (eds), Berlin: Springer. p. 49-67.

Santas, R., and P. Santas (2000). Effects of wave action on the bioremediation of crude oil saturated hydrocarbons. ***Marine Pollution Bulletin.*** 40(5): 434-439.

Shennan, J. L. (1984). Hydrocarbons as substrates in industrial fermentation. In: R.M. Atlas, (ed), ***Petroleum Microbiology.***, Macmillan, pp. 643–683

Sprovieri, M., M. L. Feo, L. Prevedello., D. S. Manta, S. Sammartino, S, Tamburrino and E. Marsella (2007). Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples habour (southern Italy). ***Chemosphere*** 67(5):998-1009.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA 4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. ***Molecular Biology and Ecology***. 24:1596-1599.

Telli-Karakoç, F., L. Tolun, B. Henkelmann, C. Klimm, O. Okay and K. W. Schramm (2002) Polyccyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) distributions in the Bay of Marmara sea: İzmit Bay. ***Environmental Pollution.*** 119:383-397.

Tso, S. F. and G. L. Taghon (2006). Protozoan grazing increases mineralization of naphthalene in marine sediment. ***Microbial Ecology*** 51:460-469.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. ***Applied Environmental Microbiology***. 73(16):5261-5267.

Weis, L. M., A. M. Rummel, S. J. Masten, J. E. Trosko, and B. L. Upham (1998). Bay and baylike regions of polycyclic aromatic hydrocarbons were potent inhibitors of gap junctional intercellular communication. ***Environmental Health Perspective***. 106(1):17-22.

West, P. A., G. C. Okpokwasili, P. R. Brayton, D. J. Grimes and R. R. Colwell (1984). Numerical taxonomy of phenanthrene-degrading bacteria isolated from the Chesapeake Bay. ***Applied Environmental Microbiology***. 48(5):988-993.

Wong, J. W. C., K. M. Lai, C. K. Wan, K. K. Ma and M. Fang (2002) Isolation and optimization of PAH-degradative bacteria from contaminated soil for PAHs bioremediation. ***Water, Air, and Soil Pollution.*** 139:1-13.

Woodhead, R. J., R. J. Law and P. Matthiessen (1999). Polycyclic aromatic hydrocarbons in surface sediment around England and Wales, and their possible biological significance. ***Marine Pollution Bulletin.*** 38(9):773-790.

Woomer, P. L. (1994). Most probable number counts. In: Weaver R. W., S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatai and A. Wollum (eds). Method of soil analysis, Part 2: Microbiological and biochemical properties. ***Soil Science Society of America***. Madison pp.59-79.

Yim, U. H., S. H. Hong, W. J. Shim, J. R. Oh and M. Chang (2005). Spatio-temporal distribution and charactheristics of PAHs in sediments from Masan Bay, Korea. ***Marine Pollution Bulletin***. 50(3):319-326 .

Zaidi, B. R., L. M. Hinkey, N. R. Rodríguez, N. S. Govind and S. H. Imam (2003). Biodegradation of toxic chemicals in Guayanilla Bay, Puerto Rico. ***Marine Pollution Bulletin.*** 46:418-423.

Zaidi, B. R. and S. H. Imam (1996). Inoculation of microorganisms to enchance biodegradation of phenolic compounds in industrial wastewater: Isolation and identification of three-indogenous bacterial strains. ***Journal of General Applied Microbiology***. 42:249-256.

Zaidi, B. R. and S. H. Imam (1999a). Factors affecting microbial degradation of polycyclic aromatic hydrocarbon phenanthrene in the Caribbean coastal water. ***Marine Pollution Bulletin***. 38(8):737-742.

Zaidi, B. R., S. H. Imam and R. V. Greene (1999b). Natural systems for better bioremediation: Isolation and characterization of a phenanthrene-utilizing strain of *Alteromonas*

from Guayanilla coastal water southwest of Puerto Rico. In ***Biopolymers: Utilizing Nature's Advanced Materials.*** Zaidi, B. R., S. H. Imam and R. V. Greene (eds). Oxford University Press. pp.204-217.

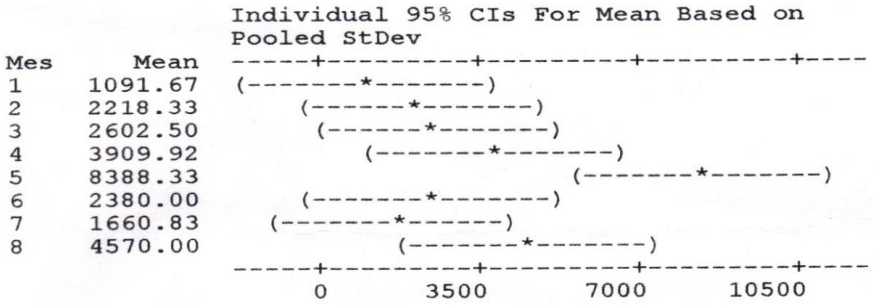
APPENDIX

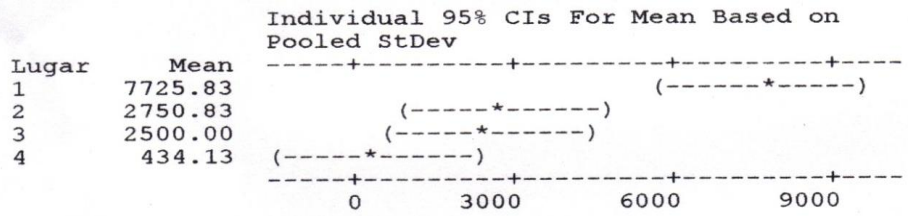
Appendix 1. Statistical results with Minitab program

Two-way ANOVA: Obs versus Mes, Lugar

Source	DF	SS	MS	F	P
Mes	7	455043965	65006281	7.06	0.000
Lugar	3	689561183	229853728	24.97	0.000
Interaction	21	1300094287	61909252	6.73	0.000
Error	64	589094607	9204603		
Total	95	3033794042			

S = 3034 R-Sq = 80.58% R-Sq(adj) = 71.18%

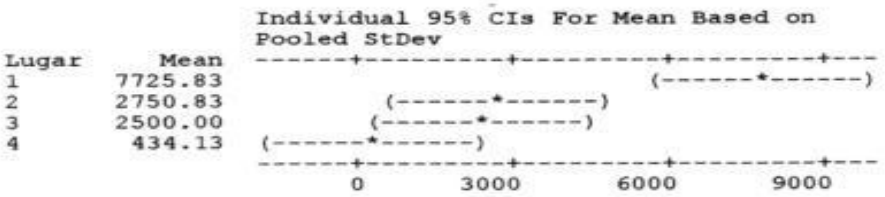


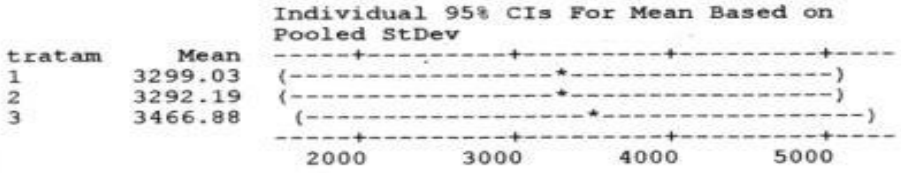


Two-way ANOVA: Obs versus Lugar, tratam

Source	DF	SS	MS	F	P
Lugar	3	689561183	229853728	8.49	0.000
tratam	2	626497	313248	0.01	0.988
Interaction	6	70496814	11749469	0.43	0.854
Error	84	2273109548	27060828		
Total	95	3033794042			

S = 5202 R-Sq = 25.07% R-Sq(adj) = 15.26%

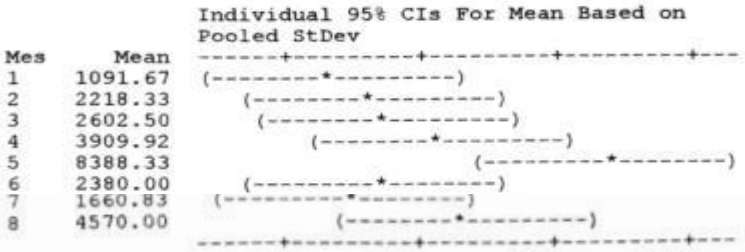


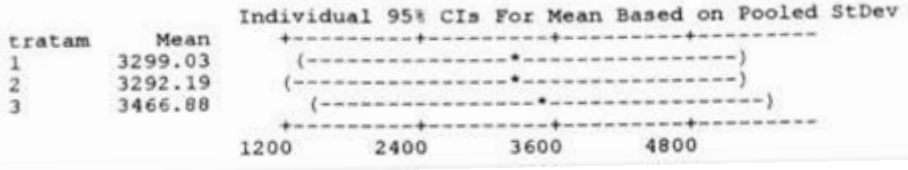


Two-way ANOVA: Obs versus Mes, tratam

Source	DF	SS	MS	F	P
Mes	7	455043965	65006281	1.95	0.074
tratam	2	626497	313248	0.01	0.991
Interaction	14	177488505	12677750	0.38	0.977
Error	72	2400635076	33342154		
Total	95	3033794042			

S = 5774 R-Sq = 20.87% R-Sq(adj) = 0.00%





Appendix 2. Mean monthly precipitation data of Guayanilla, Ponce, and Guánica stations.

	Quantity of rain (mm)		
Month	Guayanilla	Ponce	Guánica
December	0.07	0.16	0.41
January	1.59	1.21	2.16
February	0.38	0.08	0.51
March	0.99	3.18	0.97
April	1.37	0.68	1.52
May	1.84	3.70	3.22
June	1.52	1.55	0.97
July	2.44	2.17	3.35
August	1.07	2.32	2.07
September	3.29	4.57	4.08
October	1.56	2.77	2.51
November	5.10	4.26	1.72

From Atmospheric Caribbean Research Center at the University of Puerto Rico at Mayagüez

Appendix 3. Most probable number (MPN) table used for bacterial density estimation

Number of positive	Estimated Population	Number of positive	Estimated Population
1-2-3-4-5-6		**1-2-3-4-5-6**	
1-0-0-0-0-0	1.9	5-5-4-2-0-0	2159
1-1-0-0-0-0	4.0	5-5-4-3-0-0	2716
2-0-0-0-0-0	4.4	5-5-5-0-0-0	2305
2-1-0-0-0-0	6.8	5-5-5-0-1-0	3126
3-0-0-0-0-0	7.7	5-5-5-1-0-0	3282
3-1-0-0-0-0	10	5-5-5-1-1-0	4532
3-2-0-0-0-0	13	5-5-5-2-0-0	4922
4-0-0-0-0-0	12	5-5-5-2-1-0	6918
4-1-0-0-0-0	16	5-5-5-3-0-0	7797
4-2-0-0-0-0	21	5-5-5-3-1-0	10702
4-3-0-0-0-0	27	5-5-5-3-2-0	13826
5-0-0-0-0-0	23	5-5-5-4-0-0	12753
5-0-1-0-0-0	31	5-5-5-4-1-0	16902
5-1-0-0-0-0	33	5-5-5-4-2-0	21589
5-1-1-0-0-0	45	5-5-5-4-3-0	27150
5-2-0-0-0-0	49	5-5-5-5-0-0	23054
5-2-1-0-0-0	69	5-5-5-5-0-1	31225
5-3-0-0-0-0	78	5-5-5-5-1-0	32720
5-3-1-0-0-0	107	5-5-5-5-1-1	45261
5-3-2-0-0-0	138	5-5-5-5-2-0	49224
5-4-0-0-0-0	127	5-5-5-5-2-1	69148
5-4-1-0-0-0	169	5-5-5-5-3-0	78727
5-4-2-0-0-0	216	5-5-5-5-3-1	107022
5-4-3-0-0-0	270	5-5-5-5-3-2	138269
5-5-0-0-0-0	230	5-5-5-5-4-0	127528
5-5-0-1-0-0	312	5-5-5-5-4-1	169028
5-5-1-0-0-0	327	5-5-5-5-4-2	215899
5-5-1-1-0-0	453	5-5-5-5-4-3	271557
5-5-2-0-0-0	488	5-5-5-5-4-4	334051
5-5-2-1-0-0	692	5-5-5-5-5-0	230546
5-5-3-0-0-0	780	5-5-5-5-5-1	328192
5-5-3-1-0-0	1070	5-5-5-5-5-2	492238
5-5-3-2-0-0	1387	5-5-5-5-5-3	781272
5-5-4-0-0-0	1275	5-5-5-5-5-4	1312535
5-5-4-1-0-0	1690		

1 = Positive result. 0 = Negative result. Estimated population assumed 1 mL of inoculum. This factor number is adjusted by multiplying by dilution factor and inoculum volume.