Puerto Rico Water Resources & Environmetal Research Institute
Annual Technical Report
FY 2011

Introduction

The PRWRERI is an integral part of the University of Puerto Rico at Mayaguez. As such, it acts as official liaison of the University of Puerto Rico with industry and government for all water resources related research activities. The Institute also functions as a highly recognized advisor to these two sectors on water resources and environmental issues. This role translates into multidisciplinary functions and activities that add relevance and impact to the research program the Institute supports. By virtue of the local relevance of its research and the prestige and leadership of the investigators it has supported, the Institute has become the focal point for water - related research in Puerto Rico.

Projects started in FY 2011 In FY 2011, the PRWRERI strengthened collaboration with the Jobos Bay National Estuarine Research Reserve (JBNERR), located in Aguirre-Salinas, Puerto Rico. This close collaboration is supported by the Memorandum of Understanding signed by both research centers in 1998. The projects worked during 2011 included in Table 1. A short description of each project is included next:

Costal Training Program: In this project the PRWRRI provides knowledge through conferences, seminars and workshops for professional development and networking for the JBNERR staff and to enhance integration across sectors at the reserve. An Education Coordinator is in charge of this project. The project is continuing this year.

Implementation of System Wide Monitoring Program: A fundamental part of the JBNERR stormwater management program is the collection of abiotic parameters, meteorological and nutrients SWMP data. The PRWRRI was in charge of collecting, organizing, processing and submitting these data to CDMO. Data is also disseminated to the scientific community including the Research Advisory Committee for program future actions and to the Stewardship Coordinator to direct restoration efforts. The PRWRERI took charge of this project during a year, in the meantime trained personnel from the Department of Natural Resources who will be assuming a leading role this year.

Development of a Stormwater Management Plan for the Municipality of Mayagüez: Sponsored by the Municipality of Mayagüez. Part I of this project was finalized and the report submitted by October 2011.

Hydrodynamic and Salinity Study for Boquerón Wildlife Refuge: A bi-dimensional hydrodynamic and salinity model for the Wildlife Refuge Lagoon was developed using EFDC computer code. The final report was submitted in June, 2012.

Spatial works for delimiting areas for the boundaries of JBNERR: This work was used to determine the size of parcels and their impact into the JBNERR estuary. Work finished.

Taxonomy Analysis of Zooplankton and Ichthyoplankton for JBNERR: Sample collection and analysis are been performed since 2011. The study is on-going presently.

Establishment of a demonstration field in salt tolerant vegetative materials as conservation buffers in salt flats: This project started in October 2011 and has duration of three years. The project will study the effect of salt flats species in coastal erosion control. The project is a Conservation Innovation Grant from the Natural Resources Conservation Service (NRCS).

Establish surface water collector (ISCO) and collect and analyze water samples for Jobos Bay Conservation Effects Assessment Project (JBCEAP): This project is also funded by the NRCS to analyze water quality parameters from agricultural lands discharging into the Jobos Bay Reserve in Aguirre, Puerto Rico.

The Northeast States and Caribbean Islands Regional Water Program: This project is in collaboration with the University of Rhode Island is funded by the EPA. The purpose is to strengthen research, teaching and Extension capacity of Land Grand Universities to deliver outcome-based water programs that educate, empower and engage agricultural producers, residents and communities throughout the region to steward their local water resources. This project has been active for five years and is finalizing in 2012.

Outreach and technology transfer: Meetings, seminars, technical reports, quarterly newsletter and a web site are used by the Institute to keep the water resources community and general public informed about advances in research. Approximately once every three years, the Institute organizes a major conference on water-related research in Puerto Rico and the Caribbean Islands, in collaboration with other technical organizations in the region. All these activities facilitate the translation of the research sponsored by the Institute into practical applications of direct benefit to industry, government, and the general public. This year the Puerto Rico Water Resources and Environmental Research Institute Joined the Hawaii Water Resources Research Center, the Virgin Islands Water Resources Research Center and the Environmental Research Institute of the Western Pacific in Guam in organizing the conference titled Water Resource Sustainability Issues on Tropical Islands.

Also, the PRWRERI offered a two days course in River Mechanics to personnel of the Department of Natural Resources of Puerto Rico in San Juan.

Research Program Introduction

The Puerto Rico Water Resources and Environmental Research Institute (PRWRERI) is located at the Mayagüez Campus of the University of Puerto Rico. The Institute is one of 54 water research centers established throughout the United States and its territories by Act of Congress in 1964 (P.L. 88-379) and presently operating under Section 104 of the Water Research and Development Act of 1984 (P.L. 98-42), as amended. Originally, the Puerto Rico Water Resources Research Institute (PRWRRI) was established in April 22, 1965, as an integral division of the School of Engineering of the College of Agricultural and Mechanic Arts, the official name of the campus at that time. An agreement between the Director of the Office of the Water Resources Research Institute of the Department of the Interior and the University of Puerto Rico at Mayagüez was signed in May 25, 1965. This agreement allowed the Institute to receive funds as part of the Water Resources Act of 1964. In June 1, 1965, the Chancellor of the Mayagüez Campus appointed Dr. Antonio Santiago (Chago) Vázquez as the first director. The first annual allotment of funds for fiscal year 1965 was $52,297.29. Since its inception, the Institute has had eight directors in nine appointment periods as shown in the table below. Director No. - Director Name - Period of Appointment 1 Dr. Antonio Santiago-Vázquez 1965 - 1968 2 Eng. Ernesto F. Colón-Cordero 1968 - 1972 3 Eng. Felix H. Prieto-Hernández 1972 - 1974 4 Dr. Roberto Vázquez (acting director) 1974 - 1975 5 Dr. Rafael Ríos-Dávila 1975 - 1980 6 Dr. Rafael Muñoz-Candelario 1980 - 1986 7 Eng. Luis A. Del Valle 1987 - 1989 8 Dr. Rafael Muñoz-Candelario 1989 - 1994 9 Dr. Jorge Rivera-Santos 1995 - present The official name of the Institute was changed in 2005 to Puerto Rico Water Resources and Environmental Research Institute. The general objectives of the Puerto Rico Water Resources and Environmental Research Institute are (1) to conduct research aimed at resolving local and national water resources problems, (2) to train scientists and engineers through hands-on participation in research, and (3) to facilitate the incorporation of research results in the knowledge base of water resources professionals in Puerto Rico and the U.S. as a whole. To accomplish these objectives, the Institute identifies Puerto Rico's most important water resources research needs, funds the most relevant and meritorious research projects proposed by faculty from island universities, encourages and supports the participation of students in funded projects, and disseminates research results to scientists, engineers, and the general public. Since its creation, the Institute has sponsored a substantial number of research projects, supported jointly by federal, state, private, and University of Puerto Rico's funds. Through Introduction 1

The Population dynamics of the dominant Enterococci in the water systems of Puerto Rico.

Basic Information

Title:	The Population dynamics of the dominant Enterococci in the water systems of Puerto Rico.
Project Number:	2011PR129B
Start Date:	3/10/2011
End Date:	2/30/2012
Funding Source:	104B
Congressional District:	N/A
Research Category:	Water Quality
Focus Category:	Water Quality, Non Point Pollution, Ecology
Descriptors:	None
Principal Investigators:	Luis A Rios-Hernandez

Publications

1. Cuevas-Irizarry, M.F., Semidey-Rodriguez, V., Negron-Talavera, G. and, Rios-Hernandez, L.A. The dominant population of Enterococci in Puerto Rico, Poster presented at the "Water Resource Sustainability Issues on Tropical Islands", Honolulu, Hawaii, November, 2011.
2. Negron-Talavera, G., Ferguson, D.M. and Rios-Hernandez, L.A., "Environmental Enterococcus faecalis from beaches in California and Puerto Rico share a common genotype of virulence" . Poster presented at ASM 2012 San Francisco,June, 2012.
3. Mara F. Cuebas-Irizarry,M.F., Semidei-Rodríguez,V., Negrón-Talavera, G. and Ríos-Hernández, L.A., "The dominant population of Enterococci in Puerto Rico’s waterways". Poster presented at ASM 2012, San Francisco, June 2012.

Title: The Population dynamics of the dominant Enterococci in the water systems of Puerto Rico.

Principal Investigator: Luis A. Ríos Hernández [1,*]
Student contributors: Ginamary Negron (Graduate) and Mara F. Cuebas (Undergraduate)

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ABSTRACT:

BACKGROUND: The *Enterococci* are the golden standard organisms used to assess water quality in marine habitats, indicated by the regulatory agencies; as such they are used to predict fecal contamination events. But numerous scientists have questioned the utility of this group of bacteria to identify a human fecal contamination event in recreational waters. In this study we enumerated the Enterococci population and characterize the isolates biochemically, phenotypically, and genotypically. The colonies were isolated on mE, transferred to BEA, BHI with 6.5% NaCl, incubated at 45ºC, their pigmentation, catalase reaction, and motility were determined. Once those tests were all positive the isolates were subject to the molecular analysis that included the PCR amplification of the *tuf* gene, *atpA* gene and its subsequent double digestion to speciate the isolates.

RESULTS: We found that the yellow pigmented Enterococci, non-dominant in humans, dominated all waterways analyzed with 75% of the population including river and beach samples. The dominant Enterococci were identified as *E. casseliflavus* in both the river and the beach samples. In addition, *E. casseliflavus* was predominant in all seawater samples regardless of the season suggesting their adaptation to this environment when compared to the other Enterococci. Among the non-pigmented, a minor population of Enterococci in our samples (fluctuates from 14 to 23% in Añasco River while in Guanajibo river was 26 to 56%), we identified *E. faecalis* as the dominant Enterococci in all samples except the septic tanks. Actually in septic tanks we only found non-pigmented Enterococci and the most common was *E. faecium*. Furthermore, the environmental *E. faecalis* isolates from the beach or river samples contained virulence factors that were not present in any other pigmented Enterococci isolated from any of the environmental samples.

CONCLUSIONS: Taken together these results suggest that the yellow pigmented Enterococci *E. casseliflavus*, dominate in all natural water samples, but not in septic tanks. *E. faecalis*, dominant among non-pigmented, might exist or persist due to the presence of virulence factors that aid in their survival in these natural habitats. Furthermore, these isolates of *E. faecalis* could be pathogens directly or by potentially colonizing the GI tract of swimmers causing nosocomial infections in the future.

Introduction: Puerto Rico (PR) is a sub-tropical Island with an approximate population of 4 million inhabitants and an area of 9.104 km^2 and 501 km of coast line. This coastal area is enjoyed by its inhabitants for recreational use as well as by a vast number of tourists that visit P.R. each year to enjoy our warm waters year around. It is also the working place of the local fishermen which supplies the local population with fish, crustaceans, and shellfish. All these activities could be directly affected by the contamination of the water with fecal matter. In P.R. the Junta de Calidad Ambiental (JCA) is the governmental agency responsible to monitor our recreational marine waters, to do so, they use the water Fecal Indicator Organisms (FIO) suggested by the Environmental Protection Agency (EPA) which has been developed for non-tropical waters. EPA has determined that the enumeration of enterococci is the best fecal indicator organism for recreational marine environments and that it has the best relationship to swimmers illness in these waters (Cabelli, 1979; Dufour, 1984). They recommend that recreational marine waters that contain more than 35 CFU in 100 ml (geometric mean) or 104 CFU in a 100 ml in a single sample are not safe for swimmers (Dufour, 1986). Utilizing this criterion for recreational marine waters, JCA has found that all of the 24 public beaches monitored have been over the limits at least once in the last years (WWW.jca.gobierno.pr). Añasco beach (Tres Hermanos) is among the cleanest with 3 out of 23 (13%) samples been out of parameters in 2007, 16% in 2008, 12.5% in 2011, and none so far in 2012.

Since all the beaches in Puerto Rico are public and are located throughout the Island the whole population could be deprived from enjoying the recreational use of the beaches. This creates a problem to the inhabitants, the tourists, the fishermen, and ultimately to the government. It is our responsibility as scientists and inhabitants to ask the following questions: **Which species dominates in this natural environment? Which specie is the dominant one during a recent fecal contamination event? Which specie dominates in a tropical estuary and marine water sample when the sample is within parameters? How about when it is out of parameters? Do the septic tanks of the area contribute to the prevalence of the enterococci in the environment? Which specie of enterococci dominates in a septic tank from a tropical environment?**

These questions are easier to ask than to answer them since the problem is extremely complex and many physical factors could affect the quantification of the organism. These factors include water turbidity (Kay, 2005), solar radiation (Davies-Colley, 1994; Sinton, 1994), salinity (Bordalo, 2002), rainfall (Olivieri, 1977), wind direction (EPA), and tides (Desmarais, 2002; Shibata, 2004). Furthermore, the number of swimmers will also alter the quantification of fecal indicator organisms since we can shed on the order of 6 X 10^5 CFU of enterococci per person in the first 15 minutes of exposure (Elmir, 2007). In addition, recent publications suggest that *Enterococci* can survive in marine water more than 9 days (Lee, 2006) and grow in marine sediments (Lee, 2006; Desmarais, 2002; Solo-Gabriele, 2000). **But none of these publications actually attempts to differentiate among the different species of Enterococci**.

It is suggested that different species seem to have a specific host range like the case of *E. faecalis*, *E. faecium*, and *E. gallinarum* which are the species of enterococci that typically are present in human feces (Wheeler, 2002) at 10^5 to 10^7, 10^4 to 10^5 per gram of feces respectively (Fisher, 2009). It is important to realize that we still do not know which organism is the dominant species in human feces of Puerto Ricans. This is important since the distribution and dominance of Enterococci is different between countries, for example, *E. faecalis* and *E. faecium* are the most common isolated Enterococci in environmental and clinical samples in Spain and UK (Kuhn 2003). In Sweden, *E. faecium* is less common and *E. hirae* is more common, while in

Denmark *E. hirae* is dominant among slaughtered houses (Kuhn 2003). In the USA, the most common Enterococci isolated from the coastal waters and intertidal sediment in Southern California was: *E. faecalis, E. faecium, E. hirae, E. casseliflavus,* and *E. mundtii* respectively (Ferguson 2005). Interestingly, these species are also found in the feces of dogs, chickens, cows, and deer (Wheeler, 2002), in a wide variety of insects (Martin 1972), and in wild animals that include mammals, reptiles, and birds (Mundt 1963). In order to discriminate the source of these enterococci species various scientists have suggested microbial source tracking methods (Wheeler, 2002; Bahirathan, 1998) with various results but yet no consensus exist among the scientific community regarding this issue. One thing is clear, this is a very complex problem and perhaps it is due to the fact that we are using the wrong microorganism as an indicator.

To our knowledge, there are no studies that look at the dominant Enterococci in the feces of Puerto Ricans, our fauna, or our water systems, their contribution to the fecal pollution, and the population dynamics in natural environments. **More importantly, there are no studies that look at which species of Enterococci can survive and be selected within a septic tank in a tropical setting and the possibility of these organisms to colonize a tropical natural habitat such as a creek, a river, a water reservoir, an estuary, a beach or their respective sediments.** It is our intention in this report to inform your agency and the public in general our research findings that centered on our Hypothesis:

H0: Different species of Enterococci will dominate in different tropical environments (**environment selects**) such as: septic tanks, creeks/rivers, estuaries, and beach; and that these species could be used to predict accurately a recent human fecal contamination event in tropical water samples based on the differentiation of Enterococci.

Materials and Methods:

1. Enumeration of enterococci in fresh and seawater samples.

Sample collection: The sampling site will be in the west coast of the Island of Puerto Rico in the municipality of Mayaguez and Añasco. Specifically the samples will be collected at the Guanajibo River closest to the beach on the Cabo Rojo side (next to the bridge, see photo) of the river. The seawater samples were collected on the Mayaguez side of the river 30 to 50 ft from the mouth of the river and at ankle depth. The second site was the Añasco River; all the water collection was done on the Mayaguez side of the river closest to the beach. The seawater samples were collected on the Mayaguez side of the river 50 to 75 ft from the mouth of the river and at ankle depth (see photo). The sampling days, 4 times during the year including the dry season, were determined by the new moon phase, to avoid tidal differences between sampling days throughout the study. In addition, weather conditions, such as rain, storms, and water conditions will be considered prior to sampling. The sampling was done in the morning with no more than an hour difference between the two sites. A 100 ml samples were collected in sterile wide mouth bottles in triplicates. In all samplings a field blank of 100ml sterilized distilled water was brought to the sampling site and opened during the sampling and use as a negative control. Once the samples were taken, they were put on ice, transported to our laboratory, and processed immediately upon arrival. Typically the samples were processed within 3 hours of collection.

Sample processing: The water samples were processed following the USEPA Method 1600 (USEPA, 2000). The enumeration of the culturable Enterococci in the water samples was done using mEnterococcus agar (mE), after 48hrs at 41°C of incubation instead of mEI. Dark red to

maroon colonies were interpreted as presumptive Enterococci and reported as colony forming units (CFU) per 100 ml of water.

Characterization of the presumptive Enterococci: In order to confirm the presumptive status as Enterococci of the enumerated isolates, at least 10% (depending on total numbers) of the colonies were randomly chosen from the triplicate filters with a sterile toothpick and transferred to Brain Heart Infusion Agar. To confirmed purity of the picked colonies the isolates were then streaked onto BHI. Once pure colonies were obtained they were transferred to Bile Esculin Agar to determine esculin hydrolysis; to Sulfate Indol Motility media to determine motility; to Brain Heart Infusion Broth with 6.5% NaCl and also at 45°C; and using a white cotton swab pigmentation and catalase reaction were determined sequentially. Once confirmed as Enterococci, we proceeded to separate the enumerated populations into four groups based on their pigmentation and motility (See appendix 1). All incubations were done at 35°C unless otherwise stipulated.

Statistical analysis: For statistical purposes, we will take 3 samples for a representative average and the accuracy of the results. Moreover, we will use the standard deviation as an estimator to compare the variability between the samples by the enumerations of CFU/100ml that will be obtained. Confidence limits will be evaluated using the student t-test, the ranges correspond to a 95% level of confidence.

1. **To develop an effective procedure to identify the environmental Enterococci to the species level.**

All the isolates that were confirmed as Enterococci were subjected to further characterization using molecular techniques.

DNA Extraction: To extract the DNA of our isolates, we used a bead-beating method using cell biomass from overnight (12hrs of incubation) Brain Heart Infusion Broth cultures. First, we centrifuged 1.5 ml of the cultures in a microtube for 5 minutes at 13,000 rpm, the supernatant was removed and the pellet re-suspended with 200µl of 1X TE buffer (10mM Tris-HCl; 1mM EDTA, pH 8.0). Once re-suspended and homogenized, the samples were incubated at 95ºC for 10 minutes in a thermocycler to destabilize the cell membrane. Then the samples were transferred to autoclaved tubes containing 0.1mm glass beads and bead beat for 5 minutes using a vortex. Following the bead beating we centrifuge the samples for 5 minutes at 13,000 rpm and remove the supernatant to a new sterile microtube and store at -20C until used.

PCR method for enterococci identification: To identify to the genus level the Enterococci isolated from the different environmental sites we amplified 112 bp of the transcription elongation factor following the method described by Ke et al. (Ke, 1999). The identification to the species level was done by amplifying 1,102-bp of the alpha sub-unit of the ATP synthase gene (Naser, 2005) and digesting the PCR product with RsaI and AflIII to analyze a resulting Restriction Fragment Length Polymorphism (RFLP). The actual PCR protocols were modified; basically, we developed the same PCR mixture for both methods, with the exception of the specific primers for the *atpA* gene (atpA-27-R, atpA-20-F) and the *tuf* gene (Ent1, Ent2). Finally, our modified PCR mixture was composed of 22.8µL Deionized Sterile Water, 10.0 µL 10X Buffer, 5.0µL deoxynucleoside triphosphates (2.5mM each), 5.0 µL MgCl2 (25mM), 2.5 µL forward primer, 2.5 µL reverse primer, 0.2µL Promega Flexi Taq Polymerase (5U/µl) and 2.0 µL template DNA. Furthermore, the thermal cycling conditions following the Ke et al. method

was used as described; however, in the Naser et al. method we modified it the following way: denature at 95˚C for 3min; 35 cycles of 30 s at 95˚C, 30 s at 49.6˚C and 2 min at 72˚C; with a 10 min at 72˚C final extension. All PCR product sizes were confirmed by agarose-gel electrophoresis (1.8%; 111v, 90min), stained with ethidium bromide, and visualized by UV using a molecular imager system (VersaDoc MP 4000).

Double Digestion for speciation method: The PCR amplification (Naser, 2005) from the previous section served as template for the double digestion. The reaction was carried out, as follows: 20µl of *atpA* PCR product was digested with two restriction enzymes: RsaI (5U) and AflIII (5U), 5µl of 10X NE Buffer 3 and water to a final volume of 50µl (all reagents were bought at New England Biolabs). The reaction was incubated at 37°C for 2 hours and then at 80°C for 22 minutes to inactivate the enzymes using a thermocycler. Once digested the band patterns were differentiated in a 3.5% polyacrylamide-gel electrophoresis (100v, 2hrs 30min), stained with ethidium bromide, and visualized by UV using a molecular imager system (VersaDoc MP 4000).

2. To detect the presence of virulence factors among the population of environmental Enterococci.

As part of our characterization of the Enterococci isolated from our natural waters, we decided to amplify 5 different genes in a multiplex PCR protocol developed by Vankerckhoven et al. (2004). This was done in an attempt to describe the introduction of new strains of Enterococci into our natural habitats. The assay amplifies five genes that code for five virulent factors in Enterococci: aggregation substance (*asa1)*, gelatinase (*gelE)*, cytolysin (*cylA)* and enterococcal surface protein (*esp)*, and hyaluronidase (*hyl)*. Each set of primers have a characteristic product size to differentiate within the five virulence genes, for instance, *asa1* is 375bp long, *gelE* is 213bp, *cylA* is 688bp, *esp* is 510bp and *hyl* is 276bp.

Multiplex PCR method: We amplified our DNA samples from our isolated Enterococci using the Multiplex PCR method described by Vankerckhoven et al., 2004 with the following modification, instead of using the Hot-StarTaq DNA polymerase we used the Flexi Taq DNA polymerase (Promega). In all of our assays we used the DNA from *E. faecalis* strain MMH594 as positive control, which was kindly donated by Dr. Nathan Shankar, and carry four of the virulent genes (*asa1*, *gelE*, *cylA*, and *esp*). All PCR product sizes were confirmed by agarose-gel electrophoresis (1.8%, 111v, 90min), stained with ethidium bromide, and visualized by UV using a molecular imager system (BioRad VersaDoc MP 4000).

Results:

We enumerated and analyze 548 Enterococci total from both locations and water systems. We found that the yellow pigmented Enterococci, non-dominant in humans, dominated all waterways analyzed in this study (Figure 1; A and B). The pigmented Enterococci enumerated in all samples were more than 71% of the total population except in the Guanajibo River that was only 49%. Overall the motile pigmented Enterococci were 68% of the total Enterococci enumerated in all the natural habitats. The total number of Enterococci enumerated was found to be out of parameters 75% of the time (3 out of 4 sites) during the wet season and 50% of the time during the dry season. If the total number of pigmented Enterococci would be subtracted from the total number of Enterococci all the samples would be within parameters except the Guanajibo River during the wet season and Añasco River during the dry season (using 104 CFU per 100ml

for beach samples and 61 CFU per 100ml in fresh water samples). The dominant Enterococci were identified as *E. casseliflavus* in both the river and the beach samples. In addition, *E. casseliflavus* was predominant in all seawater samples regardless of the season or location suggesting their adaptation to this environment when compared to the other Enterococci. Among the non-pigmented, a minor population of Enterococci in our samples (with 14% in Añasco beach and 16% in the river while in Guanajibo was 29% in the beach and to 50.6% in the river), we identified *E. faecalis* as the dominant Enterococci followed by *E. faecium* and *E. gallinarum*. The dominant non-pigmented Enterococci were *E. faecalis* in all samples (rivers or beach) including the septic tanks. Actually in septic tanks we only found non-pigmented Enterococci in high numbers (approximately 10^5 which is 2 orders of magnitude lower than in feces, data not shown) and the most common was *E. faecium*. It is important to point out that all the information on septic tanks comes from only one septic tank.

The biochemical tests done on the isolates are summarized in Tables (Supporting Data) and could be reviewed in Figure 2. It is noticeable that 73.3% of all isolates were pigmented and only 26.7% were non-pigmented. All of the isolates were catalase negative and 98% were able to hydrolyze esculin (BEA positive) and 99% grew with 6.5% NaCl and were capable of growing at 45˚C. This screening is very effective in selecting for the right isolates and avoids the inclusion of non-Enterococci in further analysis.

Molecular Characterization

The isolates that passed the biochemical screening were further analyzed using different molecular assays to identify the presence of specific genetic material that are only found in this group of bacteria and will allow us to confirm the status as Enterococci of our isolates. One of these is the PCR amplification of the *tuf* gene which encodes for the elongation factor EF-Tu. All of the isolates that we selected from the biochemical screening were positive for the *tuf* PCR (Figure 3, see supporting data to see all the gels). Once the isolates were identified as Enterococci using the previous PCR we proceeded to ampify the α subunit of the ATP synthase gene. This PCR amplification allowed us to differentiate the isolates by simply running the amplification in an agarose gel (Figure 4, see supporting data to see all the gels). The pigmented Enterococci produce a predominant band at 300 bp (lanes 1-3, 6, 9, 11, and 13), while the non-pigmented Enterococci presented a dominant band at 1,102 bp (lanes 4, 5, 7, and 10). These different patterns help us to differentiate the pigmented from the non-pigmented Enterococci which worked to our advantage even though in theory we were expecting that all of the isolates, regardless of their pigmentation, would present only one band at 1,102 bp. In addition, these patterns were so specific to the individual isolates that we could identify it to the species level just by this technique alone (data not shown.) This was the case of *E. gallinarum* and *E. casseliflavus*.

Figure 5 shows non-pigmented Enterococci with identical pattern but it was difficult to identify specific species based on this analysis alone. These isolates required a double digestion of the PCR products in order for us to identify them to the species level (Figure 6, see Figure 40 in Supporting Data Gels for positive controls). To identify the different species of the isolated Enterococci we performed a double digestion of the ATP synthase PCR product (Figure 6). The majority of the non-pigmented Enterococci isolated showed a fragment pattern with five to six bands which is consistent with the pattern of *E. faecalis* (Figure 6) the dominant species among non-pigmented isolates from all of our water samples. The pigmented Enterococci could be identified with the *atp* PCR alone (Figure 7) and could be confirmed with the double digestion (Figure 8), lanes 1 to 7 are pigmented and lanes 9 to 18 are non-pigmented Enterococci. In figure

9, lanes 1 to 8 were pigmented Enterococci. As mentioned before the dominant pigmented Enterococci was *E. casseliflavus*, also there were other pigmented isolates but we were unable to identify them using this technique.

Likewise, with the non-pigmented, we could recognize a unique fragmentation pattern from unidentified Enterococci that was different than *E. faecalis*, *E. faecium*, and *E. gallinarum* (Figure 9). In that figure, we can observe the pattern of *E. faecalis* in lanes 2-8, the pattern of *E. gallinarum* in lane 9 and in lane 10 is the pattern of the *E. faecium*. In figure 10, we can observe the other unidentified Enterococci in lane 1, while in lanes 2, 3, 7 to 10 is *E. faecalis*, and in lanes 4 and 5 is *E. faecium*. The population size of these unidentified Enterococci was very small since we only encounter those fragmentation patterns less than five times among all of our isolates analyzed. Furthermore, the environmental *E. faecalis* isolates from the beach or river samples contained virulence factors that were not present in any other pigmented Enterococci isolated from any of the environmental samples (Figure 11).

As seen in Figure 11, only the non-pigmented Enterococci have the virulent genes (lanes 2-10), while none of the pigmented Enterococci have any of the virulent genes (lanes 12-18). Among the non-pigmented the presence of individual patterns could be used to determine the abundance and prevalence of these virulent factors among our isolates. Typically the majority of the isolates would contain two virulence factors, either gelatinase (*gel* E) and aggregation substance (*asa1*) or gelatinase and enterococcal surface protein (*Esp*). The combination of *asa1/gelE* dominated the population of non-pigmented Enterococci with 71% while *Esp/gelE* was only found in 2% of the population assayed. On the other hand, only 10% of the isolates tested (10/101) did not contain any virulent factors. The most abundant of the virulence factors was *gel E* which was found in 90% of the isolates and in 18% of those it was the only virulent factor present (see supporting evidence for details).

The dominant Enterococci isolated from septic tanks (limited to one) were identified as *E. faecium* (Figure 12). It is important to note that we only had access to one septic tank and none of the isolates contained virulence factors (Figure 13). Furthermore, we were able to demonstrate that at least one of those genotypic virulence factors is expressed and we could observe the phenotype (Figure 14). We confirmed the presence of gelatinase using the classical gelatinase test and all the isolates tested were positive in less than 4 days.

Preliminary new data:

Interestingly, a sub-sample of non-pigmented isolates from environmental habitats in PR (beach and river), California (On going collaboration with Donna Ferguson, SCCWRP), and clinical isolates from both places were analyzed for antibiotic resistance. In the isolates from PR we found that 83% of the isolates analyzed were resistant to Rifampin while they were susceptible to Piperacillin (72%). Actually all the isolates were susceptible to Piperacillin (average of 82%) regardless of their place of isolation. Surprisingly only 12% of the isolates from PR were resistant to Vancomycin while 42% of the clinical isolates from California were resistant.

Table 2. Antibiotic Resistance of isolates

Vancomycin 30µg	Resistant	Intermediate	Susceptible
PR (n=18)	12%	44%	44%
California (n=34)	9%	35%	56%
H-California (n=12)	**42%**	33%	25%
PR-Hospital (n=17)	29%	24%	47%

Rifampin 5µg	Resistant	Intermediate	Susceptible
PR (n=18)	**83%**	17%	0%
California (n=34)	50%	18%	32%
H-California (n=12)	17%	33%	55%
PR-Hospital (n=17)	29%	12%	59%

Piperacillin 100µg	Resistant	Intermediate	Susceptible
PR (n=18)	17%	11%	72%
California (n=34)	3%	0%	97%
H-California (n=12)	17%	0%	83%
PR-Hospital (n=17)	**24%**	0%	76%

Discussion

The Añasco site in both environments is dominated by yellow pigmented Enterococci with an average from both locations of 86%. At this location we found no significant difference in the population of Enterococci or the population dynamics. This finding could signify that the environment or its physico-chemical parameters are significantly similar to support this microbial population. One thing is clear, it is dominated by yellow pigmented Enterococci and the vast majority been motile suggesting the presence of *E. casseliflavus*.

In contrast, in our second location, Guanajibo, the river and the beach samples are different regarding the population and dynamics of the Eneterococci. It is noticeable that in the river the non-pigmented seem to dominate with 56% of the population while in the beach, just a few feet apart, the total population drops to around 50% of the one present in the river and is dominated by yellow pigmented Enterococci (73%) and 92.5% of those been motile representing *E. casseliflavus*. Our study suggests that at both locations in the beach the dominant Enterococci is *E. casseliflavus* not dominant in humans. This is supported by the work of (Bahirathan 1998) which found isolates of yellow pigmented Enterococci in human feces but only in one subject which was a vegetarian. Furthermore, these type of enterococci are commonly found in urban runoff (Colford 2012), wastewater treatment plants (Luczkiewicz 2010), vertebrate herbivores (Wheeler, 2002), in wild animals that include mammals, reptiles, and birds (Mundt 1963), and even in insects (Martin 1972) suggesting a non-human origin. In addition other investigators have found them associated with aquatic plant material and forming biofilms on different submerged surfaces (Badgley 2010). It is important to notice that the Ganajibo River was dominated by non-pigmented Enterococci which the majority was identified as *E. faecalis* a common organism associated with human feces. Interestingly the dominant Enterococci found in septic tanks was *E. faecium* which did not dominated in any of our sampling sites suggesting that perhaps these organisms do not make it to these rivers and beaches and if they do they are eliminated or outcompeted by other species.

The methodology used in this study proof to be excellent in screening for Enterococci and reducing the inclusion of non-Enterococcal isolates in our study. It is important to notice that this methodology would take approximately 5 days for the biochemical characterization and 4 days for the molecular analysis for a maximum of 10-20 isolates. Even though this approach is time consuming the end results justify the work, especially when we are trying to understand dominance, speciation, and genotipically characterize the isolates instead of just suggesting fecal pollution.

The molecular characterization utilizing the *tuf* gene proof to be a reliable method with one hundred percent of the isolates been identified as Enterococci. Of course we did not really test the accuracy of the primers (already published, Ke et. al.) since any isolate that we submitted to this analysis was already screened by our biochemical characterization which included selective and differential growth media. Perhaps the length of our analysis could be reduced to 4 or 5 days if we eliminate the biochemical characterization, but again we were not interested in developing a faster and better method to assess water quality, we were interested in identifying the dominant species of Enterococci in our waterways. To accomplish this we used the best phylogenetic marker for the genus Enterococci which is the alpha sub-unit of the ATP synthase gene (Naser, 2005). This gene has more resolution power than the commonly used 16S rRNA gene (Naser, 2005). Our primer set was suppose to amplify a fragment of 1,102 bp from all Enterococci and to our surprise the size and number of fragments amplified were different depending on the Enterococci and the DNA polymerase used (data not shown). Instead of this

been a limitation it actually turned out to be an asset because it allowed us to differentiate certain species from the PCR products directly. Furthermore, it allowed us to differentiate the pigmented Enterococci from the non-pigmented ones in just one step. Unfortunately, this set of primers is not specific to the Enterococci and could not be used in any other format such that it would allow us to eliminate the isolation step and still identify the different Enterococci present in a particular sample.

The identification to the species level was achieved by digesting the ATP PCR product with two endonuleases (RsaI and AflIII) this resulted in a unique RFLP pattern for each species. We were able to identify clearly the dominant pigmented Enterococci, *E. casseliflavus*, and among the non-pigmented Enterococci we identify *E. faecalis*, *E. faecium*, and *E. gallinarum* in our natural samples. In general, we were able to identify all the pigmented Enterococci (*E. pallens*, *E. gilvus*, E. *mundtti*, and *E. sulfureus*) and some of the non-pigmented (*E. aquimarinus*, *E. durans*, *E. hirae*, *E. avium*) in addition to the previously mentioned Enterococci. We feel confident that we would be able to identify the majority if not all the Enterococci described to date using this method, but we did not have all the representative organisms to test our method. Furthermore, this method is fast and relatively cheap once you have the DNA of the isolates when compare to similar speciation methods like Jackson et. al. (2004). This method relies on a multiplex PCR and requires 7 different PCR reactions and 56 primers to identify all the species. This approaches although strait forward could be very expensive especially when you are trying to identify Enterococci without any previous suggestion of the possible species in the samples. Our method only requires one set of primers, the subsequent double digestion, and the separation of the fragments in a polyacrylamide gel. One thing to consider in our method is the interpretation of our results could be very difficult if the individual isolates ran in a gel are diverse, thus we suggest to reorganize the gels with isolates that produce the same pattern and this will simplify the analysis.

Interestingly, we found that *E. casseliflavus*, a yellow pigmented Enterococci, was dominant in all water samples except in Guanajibo River and septic tanks. Actually in septic tanks we only found *E. faecium* exclusively. It is worthwhile noticing that we only had access to one septic tank so to conclude without reservations would be wrong. Having said that, this preliminary finding was surprising for two reasons; first the septic tanks are blamed for the contamination found in Puerto Rico's water ways and second in our samples we only found *E. faecium* in less than 5% of the isolates. This suggest a few interesting possibilities; it is possible that *E. faecium* is the dominant Enterococci in feces of Puerto Ricans, or that they are favored within septic tanks, either way further analysis is required to make valid conclusions.

Among the non-pigmented Enetreococci, although not dominant, the natural environments were heavily dominated by one species of Enterococci, *E. faecalis.* Using the double digestion allowed us to accurately identify all the different isolates as *E. faecalis* since we were using a phylogenetic marker (*atpA* PCR product) but we could not differentiate among two *E. faecalis* from different environments. To do that we use the virulence factor multiplex PCR. Utilizing this technique we could discriminate among strains of *E. faecalis* genotypically and identify at least four different genotypes present in all samples except in the septic tanks (Eaton 2001. Among these genotypes the dominant one was the combination of *asa1* and *gelE* while the most common virulent factor among Enterococci was *gelE*. The relevance of these findings will require more studies, but we could suggest that these virulent genes could help the Enterococci survive and persist in the aquatic environment. For instance, aggregation substance (*asa 1*) is an inducible plasmid bound protein that promotes the clumping of cells to favor conjugation (Galli 1990), as a

virulence factor it increases adherence to human cells in the heart, intestines, and renal cells (Guzman, 1989). In the environment it could play an important role in adherence to sand particles or aquatic plants by the formation of a biofilm. *Gel E* is a chromosomal gene that encodes an extracellular protein with endopeptidase activity and uses as substrates collagen, gelatin, and small peptides (Su, 1991). In a clinical scenario this protein actually increases the severity of disease in different animal models (Engelbert 2004, Mylonakis 2002). In natural environments this protein might allow the Enterococci to access different carbon sources to gain energy and survive for longer periods of time. The combination of both proteins could indeed work synergistically to improve the survivability and prevalence of these isolates in natural habitats. We suspect that these Enterococci might have a human origin but at this point of our investigation we are not certain that this is the case especially with our preliminary results from the septic tanks. The actual relation between these environmental isolates and the clinical isolates that cause diseases in humans is not clear at this moment (Moellering 1992). We still do not know, for sure, if these environmental isolates that contain these virulent factors are actually capable of causing an illness in humans.

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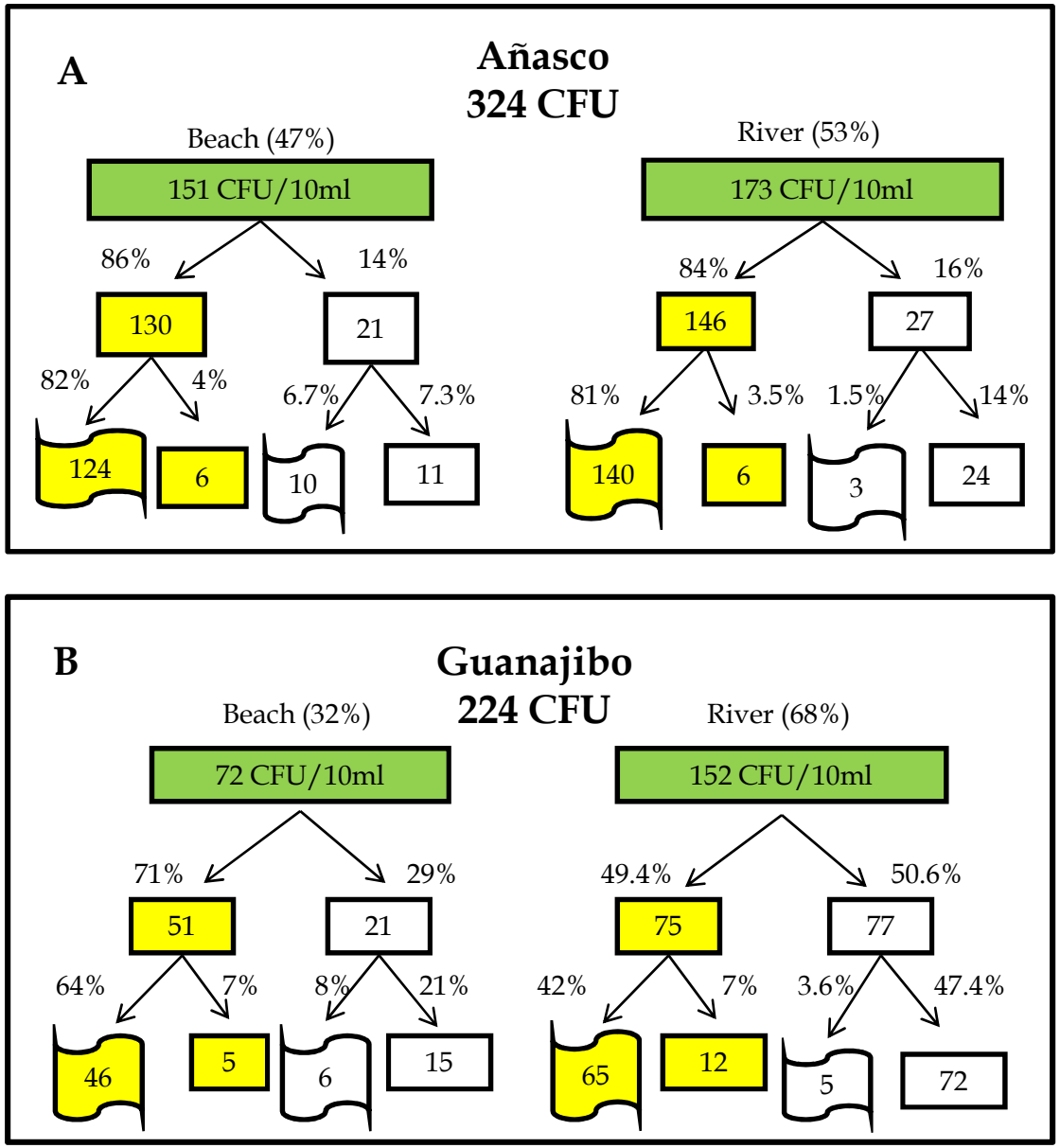
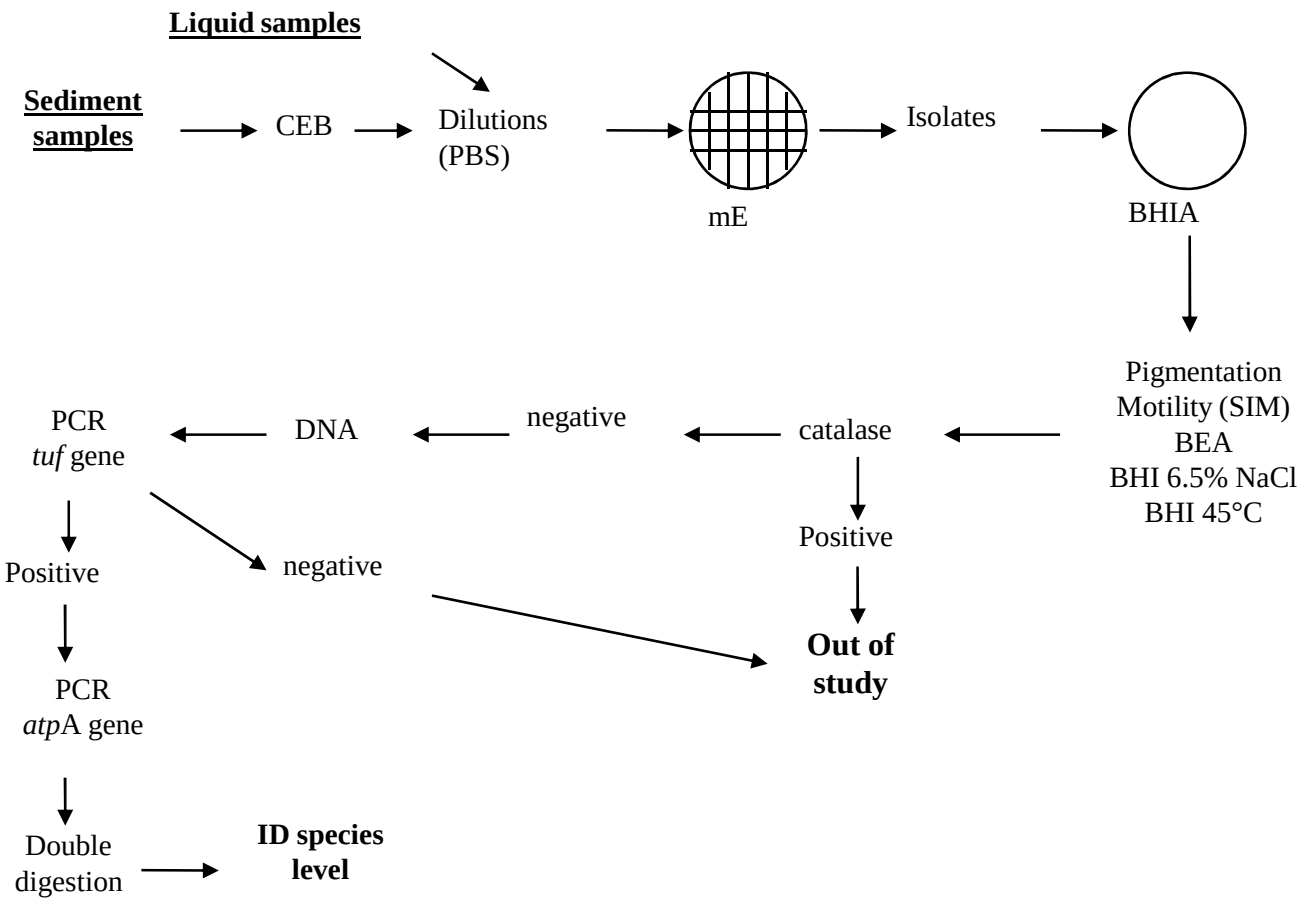


Figure 1. Enumeration of environmental Enterococci using the membrane filtration method on mE agar. Pigmentation was determined using an isolated colony from a plate of BHI agar 24 hours after transferred from the filter on mE agar. The colony was picked up with a white cotton swab to visualize yellow pigmentation. The motility was determined after 24 hours of incubation in SIM medium. All incubations were done at 37˚C except mE which was done at 41˚C for 48 hours. A) Añasco site, river and beach samples. B)Guanajibo site, river and beach samples.

Figure 2. Flow chart of methodology



PCR *tuf* gene - Añasco River

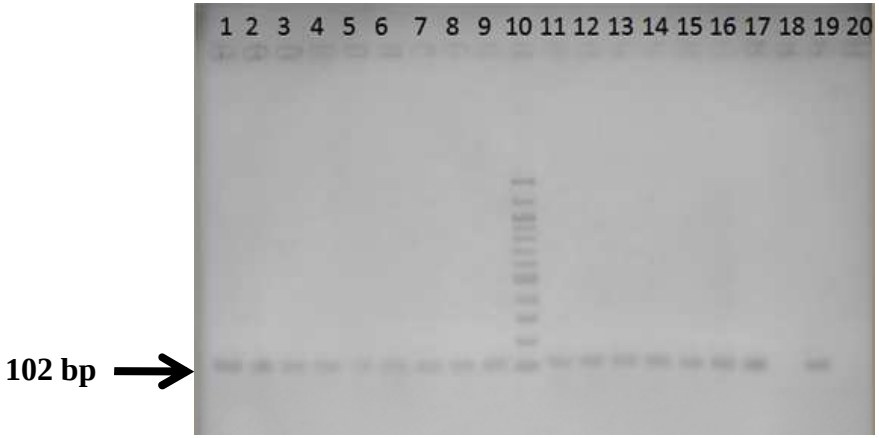


Figure 3. PCR amplification of *tuf* gene from Añasco River isolates. Lane 1, R1; lane 2, R2; lane 3, R3; lane 4, R4; lane 5, R5; lane 6, R6; lane 7, R7; lane 8, R8; lane 9 R9; lane 10, molecular marker (100bp); lane 11, R10; lane 12, R11; lane 13, R12; lane 14, R13; lane 15, R14; lane 16, R15; lane 17, R16; lane 18, empty; lane 19, *E. faecalis* (positive control); lane 20, negative control (no DNA added). Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

PCR amplification of *atpA* gene –from Añasco River/Beach

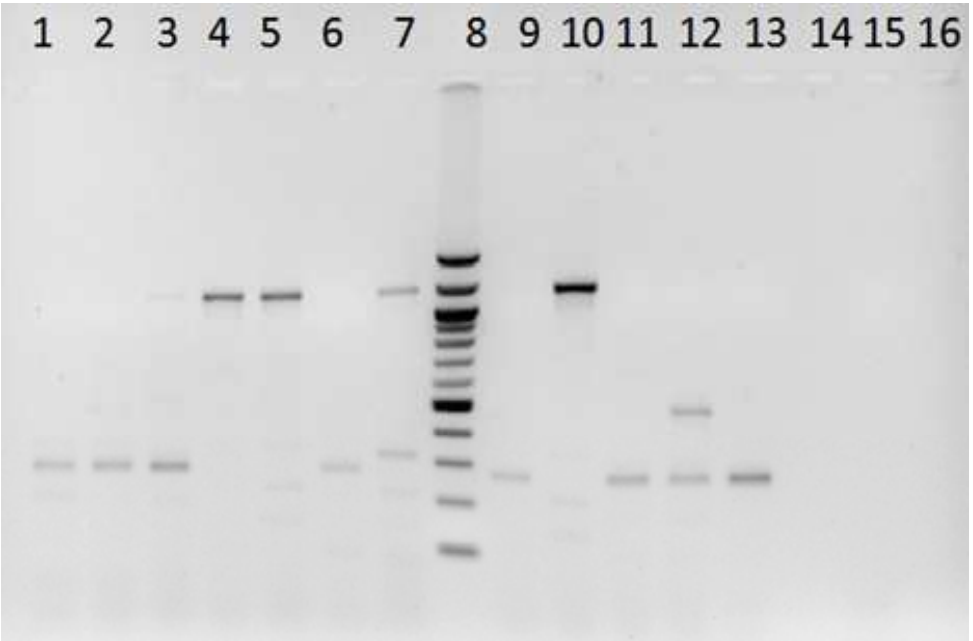


Figure 4. PCR amplification of *atpA* gene of isolates from Añasco site. Lane 1, R29 (River); lane 2, R30 (River); lane 3, R31 (River); lane 4, P1 (Beach); lane 5, P2 (Beach); lane 6, P3 (Beach); lane 7, P4 (Beach); lane 8 (Beach), molecular marker (100bp); lane 9, P5 (Beach); lane 10, P6 (Beach); lane 11, P7 (Beach); lane 12, P8 (Beach); lane 13, P9 (Beach); lane 14, empty; lane 15, negative control (no DNA added); lane 16, empty. Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

PCR of *atpA* gene of non Pigmented Enterococci isolated from Guanajibo River

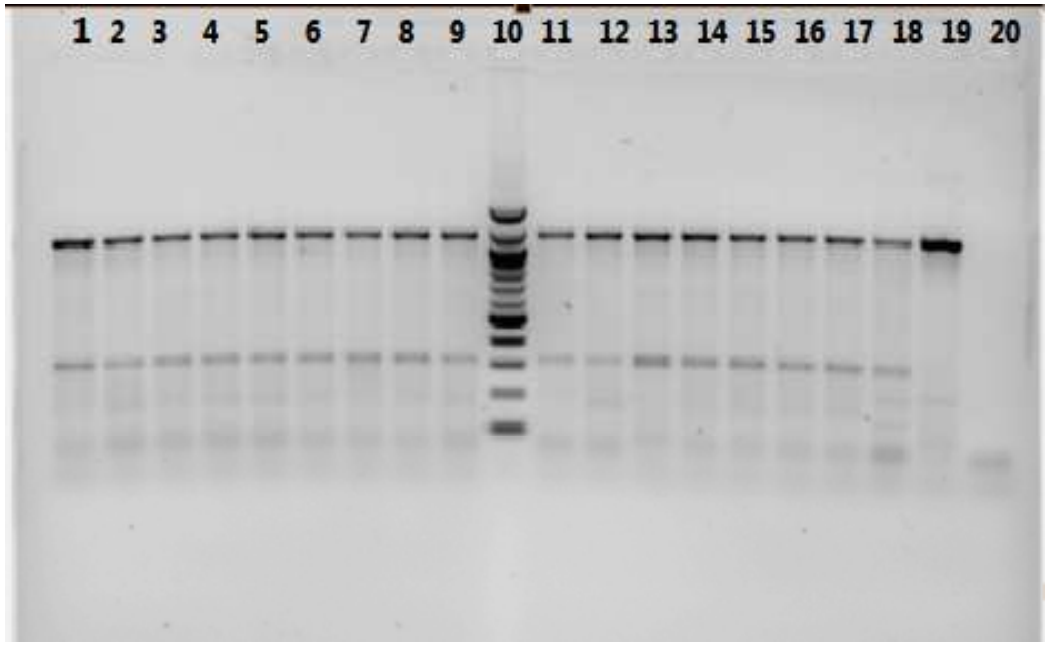


Figure 5. *atpA* gene PCR of non pigmented isolates-Guanajibo River. Lane 1, RG1; lane 2, RG5; lane 3, RG9; lane 4, RG13; lane 5, RG17; lane 6, RG18; lane 7, RG19; lane 8, RG20; lane 9, RG21; lane 10, molecular marker (100bp); lane 11, RG22; lane 12, RG23; lane 13, RG24; lane 14, RG25; lane 15, RG26; lane 16, RG27; lane 17, RG28; lane 18, Positive Control (*E. faecalis)*; lane 19, Positive Control (*E. faecium);* lane 20, Negative Control (No DNA added). Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

RFLP of ATP Synthase gene of non -Pigmented Enterococci from Guanajibo River

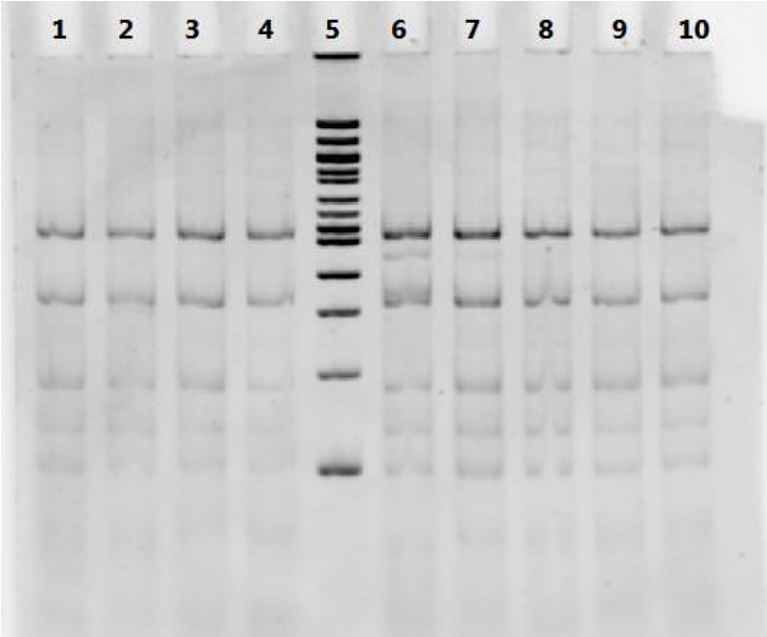


Figure 6. RFLP of *atpA* gene PCR product of Guanajibo River-Non Pigmented Isolates. Lane 1, RG20; lane 2, RG19; lane 3, RG21; lane 4, RG22; lane 5, molecular marker (100bp); lane 6, RG24; lane 7, RG25; lane 8, RG26; lane 9, RG27; lane 10, RG28. Electrophoresis conditions: 3.5% polyacrylamide gel/100V/2h 30 min, inverted stained with ethidium bromide.

atpA gene PCR- Guanajibo River
(Pigmented and Non Pigmented)

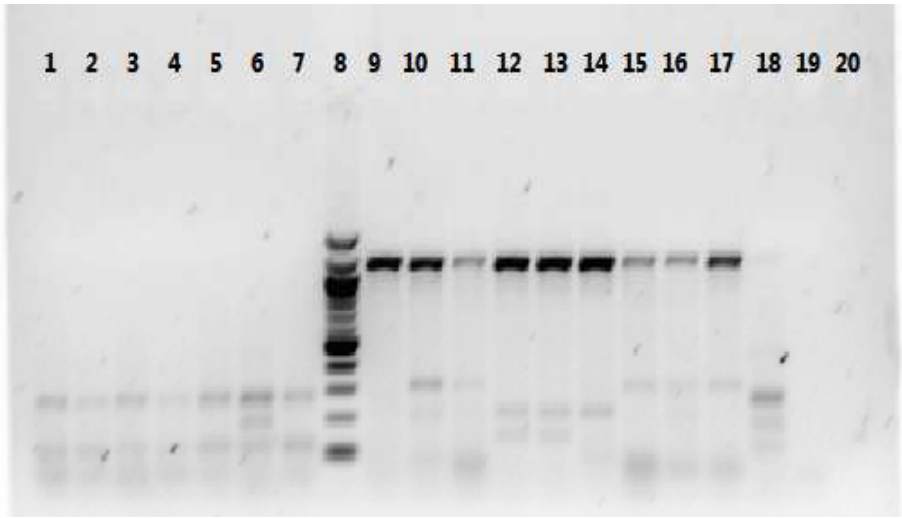


Figure 7. *atpA* gene PCR of Guanajibo River isolates. Lanes 1-7 (Pigmented Isolates). Lane1, IRG25; lane 2, IRG27; lane 3, IRG29; lane 4, IRG31; lane 5, IRG32; lane 6, IRG33; lane 7, IRG34; lane 8, molecular marker (100bp); Lanes9-19 (Non Pigmented Isolates). Lane 9, IRG7; lane 10, IRG8; lane 11, IRG18; lane 12, IRG21; lane 13, IRG21; lane 14, IRG22; lane 15, IRG26; lane 16, IRG28; lane 17, IRG30; lane 18, IRG19; lane 19, Negative Control (No DNA added)*;* lane 20, empty. Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

RFLP of *atpA* gene PCR product of Añasco Beach Pigmented Isolates.

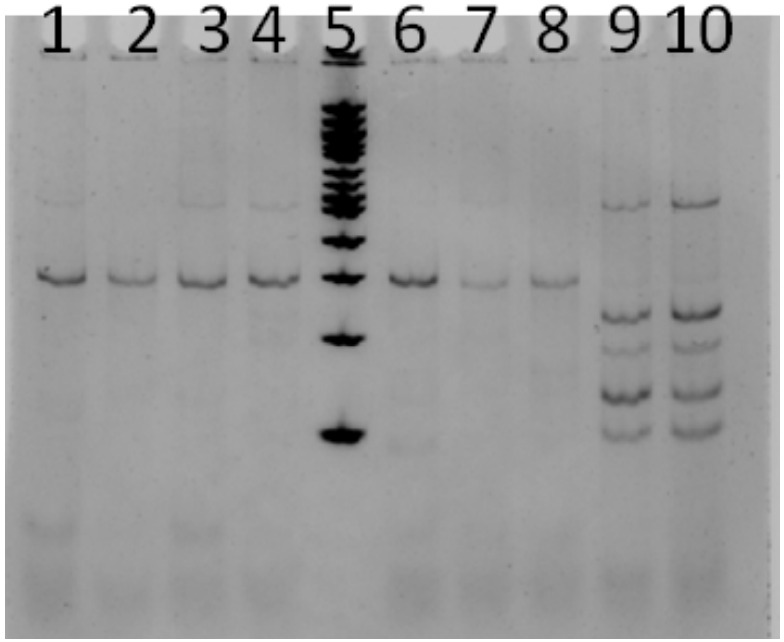


Figure 8. RFLP of *atpA* gene PCR product of Añasco Beach Pigmented Isolates. RFLP of *atpA* gene PCR of. Lane 1, P3; lane 2, P5; lane 3, P7; lane 4, P9; lane 5, molecular marker (100bp); lane 6, P1; lane 7, P2; lane 8, P4; lane 9, P6; lane 10, P8. Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

RFLP of *atpA* gene PCR of Environmental-Non Pigmented Isolates

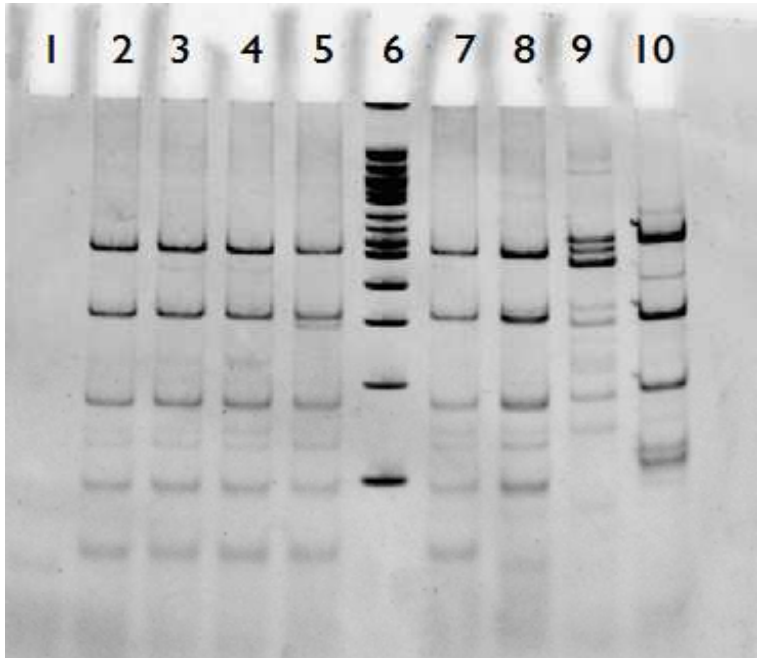


Figure 9. RFLP of *atpA* gene PCR of Environmental-Non Pigmented Isolates. Lanes 1-7 (Añasco River). Lane1, IRA4; lane 2, IRA8; lane 3, IRA12; lane 4, IRA15; lane 5, IRA24; lane 6, molecular marker (100bp); lane 7, IRA32; lane 8, IPA3 (Añasco Beach); lane 9, IPA8 (Añasco Beach); lane 10, IPG3 (Guanajibo Beach). Electrophoresis conditions: 3.5% polyacrylamide gel/100V/2h 30 min, inverted stained with ethidium bromide.

RFLP of *atpA* gene PCR of Guanajibo River-Non Pigmented Isolates

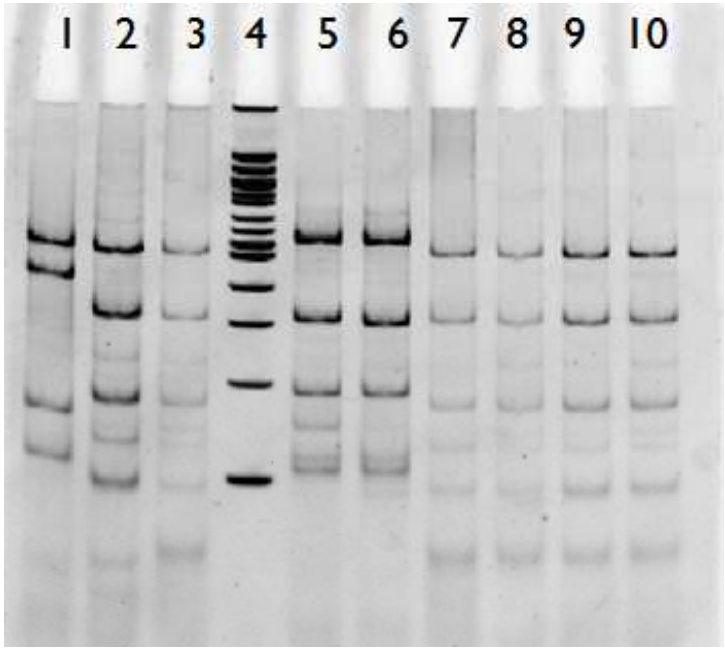


Figure 10. RFLP of *atpA* gene PCR of Guanajibo River-Non Pigmented Isolates. Lane 1, IRG7; lane 2, IRG8; lane 3, IRG18; lane 4, molecular marker (100bp); lane 5, IRG21; lane 6, IRG22; lane 7, IRG26; lane 8, IRG28; lane 9, IRG30; lane 10, IRA1 (Añasco River). Electrophoresis conditions: 3.5% polyacrylamide gel/100V/2h 30 min, inverted stained with ethidium bromide.

Virulence Factors of Environmental Isolates

(Pigmented vs. No Pigmented)

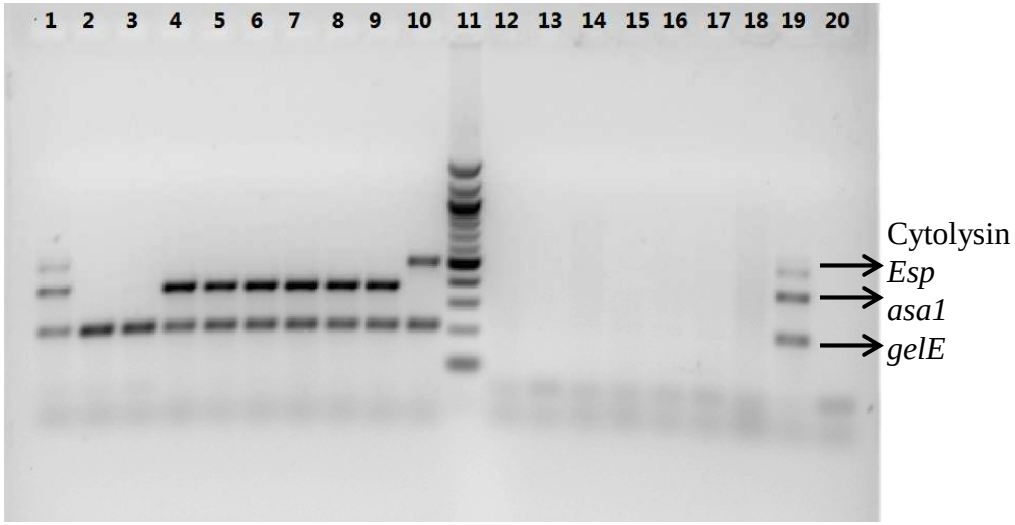


Figure 11. Multiplex PCR of Virulence Factors. Lane 1, *E. faecalis* MMH594 (positive control for *asa1*, *gelE*, *cylA*, and *esp*). Lanes 2-10 (Non Pigmented Isolates). Lane 2, RG24; lane 3, RG22; lane 4, RG34; lane 5, RG39; lane 6, RG35; lane 7, RG29; lane 8, RG36; lane 9, RG19; lane 10, PG5; lane 11, molecular marker (100bp). Lanes 12-18 (Pigmented Isolates). Lane 12, PA7; lane 13, PA16; lane 14, PA17; lane 15, PA11; lane 16, PA10; lane 17, PG10; lane 18, PA13; lane 19, *E. faecalis* MMH594 (positive control for *asa1*, *gelE*, *cylA*, and *esp*); lane 20, Negative Control (No DNA added). Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

RFLP of *atpA* gene PCR of non pigmented isolates-Septic Tanks

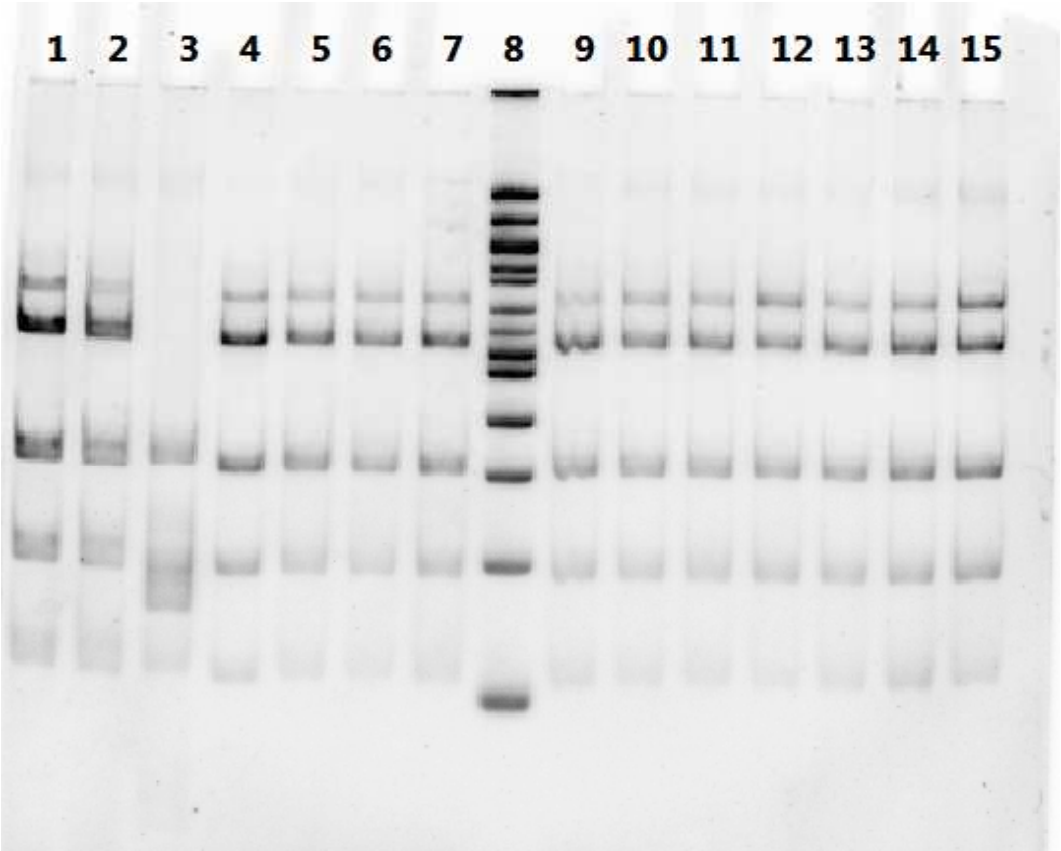


Figure 12. RFLP of *atpA* gene PCR of non pigmented isolates-Septic Tanks. Lane 1, A71; lane 2, A72; lane 3, A73; lane 4, A75; lane 5, A76; lane 6, A78; lane 7, A79; lane 8, molecular marker (100bp); lane 9, A94; lane 10, A95; lane 11, A96; lane 12, A97; lane 13, A98; lane 14, A99; lane 15, A100. Electrophoresis conditions: 3.5% polyacrylamide gel/111V/2h 30 min, inverted stained with ethidium bromide.

Multiplex PCR of Virulence Factors Non-pigmented isolates of Septic Tanks.

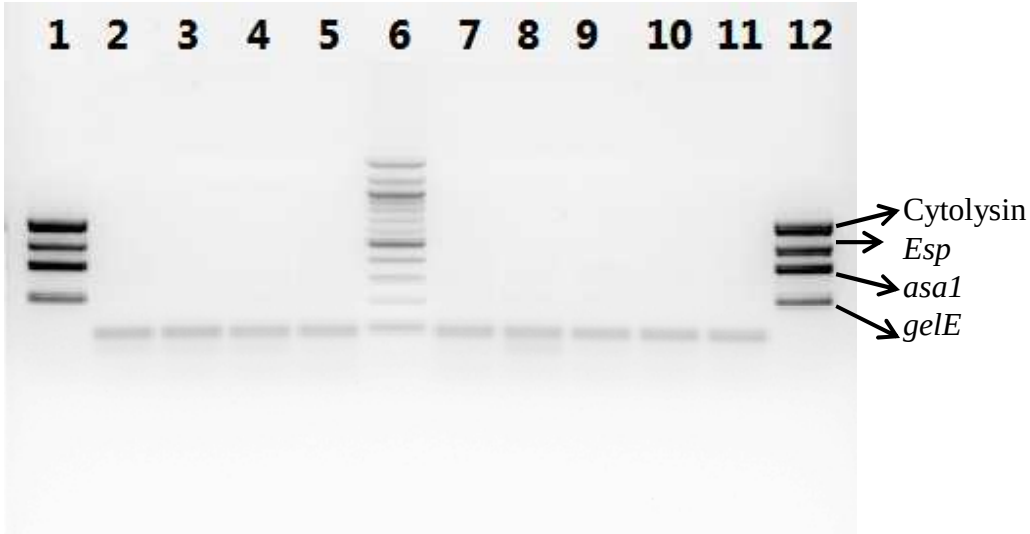


Figure 13. Multiplex PCR of Virulence Factors-Non pigmented isolates of Septic Tanks. Lane 1, *E. faecalis* MMH594 (positive control for *asa1*, *gelE*, *cylA*, and *esp*); lane 2, A71; lane 3, A72; lane 4, A73; lane 5, A74; lane 6, A75; lane 7, molecular marker (100bp); lane 8, A76; lane 9, A78; lane 10, A79; lane 11, *E. faecalis* MMH594 (positive control for *asa1*, *gelE*, *cylA*, and *esp*); lane 12, Negative Control (No DNA added). Electrophoresis conditions: 1.8 agarose/111V/1h 30 min, inverted stained with ethidium bromide.

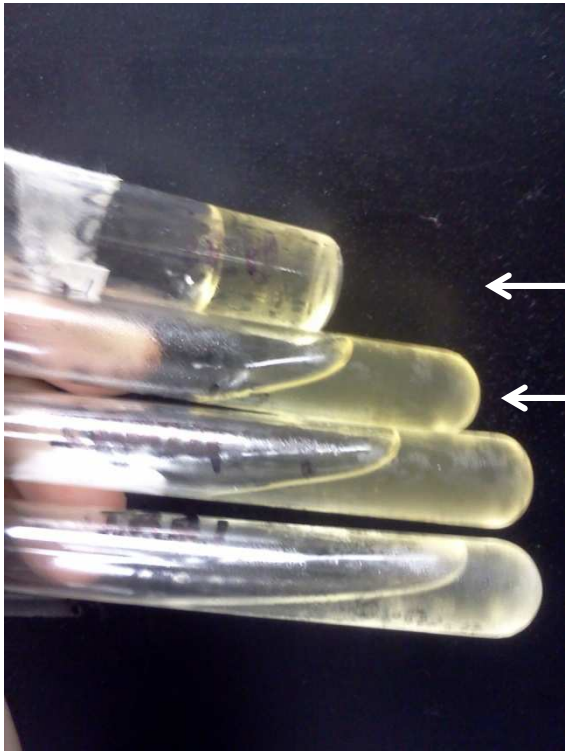


Figure 14. Gelatinase assay

Information Transfer Program Introduction

The Puerto Rico Water Resources and Environmental Research Institute joined the Hawaii Water Resources Research Center, the Virgin Islands Water Resources Research Center and the Environmental Research Institute of the Western Pacific in Guam in organizing the conference titled Water Resource Sustainability Issues on Tropical Islands.

Island Institute Directors' s Conference/Workshop

Basic Information

Title:	Island Institute Directors' s Conference/Workshop
Project Number:	2011PR132B
Start Date:	3/1/2011
End Date:	2/29/2012
Funding Source:	104B
Congressional District:	N/A
Research Category:	Not Applicable
Focus Category:	Water Quality, Water Quantity, Water Supply
Descriptors:	None
Principal Investigators:	Jorge Rivera-Santos, Walter Silva

Publication

1. Abstracts of the "Water Resource Sustainability Issues on Tropical Islands", Honolulu, Hawaii, Nov. 14-16, 2011. (Digital file)

Conference on
Water Resource Sustainability Issues on Tropical Islands

University of Hawaii, Nov. 14-16, 2011

The Puerto Rico Water Resources and Environmental Research Institute joined the Hawaii Water Resources Research Center, the Virgin Islands Water Resources Research Center and the Environmental Research Institute of the Western Pacific in Guam in organizing the conference titled ***Water Resource Sustainability Issues on Tropical Islands.***

Researchers, students, managers and other professionals working in environmental problems in the tropical region joined in Honolulu, from November 14 to 16, 2011. The directors from all four island regional water centers; as well as, researchers and students from the Pacific and the Atlantic side of the world attended the activity. The Conference was an excellent opportunity for interaction of persons from the academia, industry and government in discussing issues related to water quantity and water quality, with special focus on the Islands. This interaction was particularly significant due to geographical and political separation between the Islands represented at the Conference.

Islands around the world have unique ecosystems and are prone to natural hazards such as flash floods, landslides, contamination and tsunamis. They also have major challenges in water supply and wastewater treatment. Bigger islands have superficial and groundwater sources of water, however, small islands are severely limited in water sources and must rely on rainfall harvesting, expensive desalination or importing water. Some of these sources, such as desalination, represent a high cost on water and may limit water availability for part of the population. Wastewater treatment and disposal, as well as, non-point source pollution also have significant impacts on health, nutrition and the tourism industry, which is a very important contribution to local economies.
The presentations, keynote speaker sessions and round tables offered at this conference promoted interaction among the scientific, technical and governmental communities to discuss and propose ideas contributing to the solution of these problems.

The topics covered in the Conference were:

Wastewater
Flooding
Climate
Water Resources Availability and Management
Groundwater Recharge
Surface Water Quality
Water for Energy
Submarine Groundwater Discharge
Groundwater Quality

Approximately 128 participants from Puerto Rico, the U.S. Virgin Islands, Guam, American Samoa, Hawaii and other islands in the pacific were benefited from this activity which counted a total of approximately 50 presentations plus some panel discussions on related topics. Besides been an excellent opportunity to strengthen relations among professionals from different parts of the world it also produced the electronic publication of the conference proceedings available at

https://sites.google.com/site/wrrcconference2011/Home

The presentations are indicative of the efforts been made to improve the situation of the water resources in the tropical islands.

USGS Summer Intern Program

None.

Student Support					
Category	**Section 104 Base Grant**	**Section 104 NCGP Award**	**NIWR-USGS Internship**	**Supplemental Awards**	**Total**
Undergraduate	2	0	0	9	11
Masters	2	0	0	5	7
Ph.D.	1	0	0	0	1
Post-Doc.	0	0	0	0	0
Total	5	0	0	14	19

Notable Awards and Achievements