

**Puerto Rico Water Resources & Environmental
Research Institute
Annual Technical Report
FY 2012**

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 (PRWRRRI) 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 (3 yr) 2 - Eng. Ernesto F. Colón-Cordero - 1968 - 1972 (4 yr) 3 - Eng. Felix H. Prieto-Hernández - 1972 - 1974 (2 yr) 4 - Dr. Roberto Vázquez (acting director) - 1974 - 1975 (1 yr) 5 - Dr. Rafael Ríos-Davila - 1975 - 1980 (5 yr) 6 - Dr. Rafael Muñoz-Candelario - 1980 - 1986 (6 yr) 7 - Eng. Luis A. Del Valle - 1987 - 1989 (2 yr) 8 - Dr. Rafael Muñoz-Candelario - 1989 - 1994 (5 yr) 9 - Dr. Jorge Rivera-Santos - 1995 present (18 yr)

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 its website, the Institute's work is more widely known to the Puerto Rican and world communities and, at the same time, provides means of information transfer with regard to the reports produced through the institute's research activities.

The Institute is advised by an External Advisory Committee (EAC) composed of members from water resources related government agencies, both federal and state levels. This committee virtually convenes annually to established research priorities and to evaluate and recommend proposal for funding under the 104-B program. The EAC has representation from the private sector as well. The FY-2012 EAC composition was as follows.

1. Dr. Antonio Santiago Vázquez, Engineering Consulting Firm, former Institute's director. 2. Mr. Pedro Díaz, USGS District Chief, Puerto Rico and Caribbean Office. 3. Eng. Victor Trinidad, US Environmental Protection Agency 4. Eng. Angel Meléndez, PR Environmental Quality Board 5. Dr. Walter Silva, Associate

Director, PRWRERI, UPRM 6. Dr. Jorge Rivera-Santos, Director, PRWRERI, UPRM

Due to the close retirement dates of some of the EAC members, the Institute's Director will engage in recruiting new members for next fiscal year. New agencies that could participate in the EAC include the PR Department of Natural and Environmental Resources (PRDNER), Federal Emergency Management Agency (FEMA), US Fish and Wildlife Service (FWS) and US Army Corps of Engineers (CoE).

This report covers the period from March 1, 2012 to February 28, 2013. All activities relate to the base grant, National Competitive Grant Program awards for which the Institute was the lead institute, NIWR-USGS Internships, and supplemental awards funded by either the USGS or by pass-through funds from another Federal agency are summarized herein.

Research Program 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.

FY-2012 104-B base grant supported three research projects, one new and two continuing projects. The project *An Integrated Approach for the Detection of Estrogenic Activity in a Tropical Urban Watershed*, in charge of Dr. Jorge Ortiz, a PI from the Río Piedras Campus of the University of Puerto Rico system, aims to assess the presence of estrogenic activity in freshwater environments in Puerto Rico affected by human activities. The second and third projects are continuing projects by PIs from our home campus. These are

Microbial Source Tracking: The hunt for E. faecalis the dominant Enterococci among non-pigmented environmental Enterococci in the water systems of Puerto Rico by Dr. Luis Rios and *Field Demonstration of Removal of MS2 Bacteriophage and Bacillus subtilis with a Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit* by Dr. Sangchul Hwang.

In FY 2012, the PRWRERI continued strengthening its 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 organizations in 1998. A short description of each undergoing (or ended during FY-2012) project is included next.

Coastal Training Program (CTP): 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. This project was renewed during FY-2012 and continues through September 2013. Among the conferences held this year are *Collection and Mounting of Arthropods* and *Birds Banding and Molting Techniques*. The CTP focuses on (a) green design and sustainable principles incorporated in infrastructure planning and/or operational procedures to address climate change and resource protection, (b) socio-economic sustainability and community empowerment geared towards coastal resource protection, and (c) community and ecosystem resilience related to natural disasters.

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 the Centralized Data Management Office (CDMO). Data is also disseminated to the scientific community including the JBNERR's 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 ending in December, 2012. As part of the project, personnel from the PR Department of Natural and Environmental Resources were trained and have assumed a leading role in data collection for the reserve.

Spatial works for delimiting areas for the boundaries of JBNERR: This work was used to determine the size and boundaries of parcels surrounding the reserve. Construction on some of these parcels has negatively impacted the ecosystem of the JBNERR estuary. This work was finished in August, 2012.

Taxonomy Analysis of Zooplankton and Ichthyoplankton at JBNERR: This is a prospective zooplankton taxonomic composition and abundance monitoring program launched by JBNERR administration as part of

Research Program Introduction

their System-Wide Monitoring program (SWMP) targeting biotic, abiotic, and land use/habitat change aspects of the Jobos Bay estuary. Sample collection and analysis are been performed since 2011. The project was renewed during FY-2012 with new termination date as April, 2014.

A new educational and training project for JBNERR was started on December, 2012. The JBNERR Training and Technology Transfer for School Teachers (3TST) aims to provide schools from the Municipality of Guayama and Salinas with training, technology transfer, and information on water quality and environmental issues. This is a collaborative effort between PRWRERI, JBNERR, and OPAS (Spanish acronyms for the Organization Pro Environmental Sustainability) to promote education programs among schools in the vicinity of JBNERR. In a parallel effort, started on November 2012, PRWRERI continue supporting the JBNERR programmatic strategic plan by analyzing geospatial environmental data. These data relates to the land use and habitat inventory and looks for areas subjected to possible susceptibility to environmental problems.

Collaboration with other federal and state agencies has resulted in various externally funded projects. The Puerto Rico Department of Natural and Environmental Resources provided funds for the Hydrodynamic Modeling and Salinity Study for Boquerón Wildlife Refuge project. 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.

On the other hand, the Natural Resources Conservation Service (NRCS) of the Department of Agriculture supported the 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 studies the effect of salt flats species in coastal erosion control. The project is a Conservation Innovation Grant. Another project sponsored by NRCS is the Establishment of surface water collector (ISCO) and collection and analysis of water samples for Jobos Bay Conservation Effects Assessment Project (JBCEAP). This project analyzes stormwater 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, in collaboration with the University of Rhode Island, was funded by EPA. The purpose was to strengthen research, teaching, and extension capacity of Land Grant 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 was active for five years and finished in September, 2012.

Field Demonstration of Removal of MS2 Bacteriophage and Bacillus subtilis with a Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit

Basic Information

Title:	Field Demonstration of Removal of MS2 Bacteriophage and Bacillus subtilis with a Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit
Project Number:	2012PR136B
Start Date:	3/1/2012
End Date:	2/28/2014
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Water Quality, Water Supply, Treatment
Descriptors:	
Principal Investigators:	Sangchul Hwang

Publication

1. Hwang, S., D. Concepción, M. Hernandez, 2012. Solar-powered engineered experimental drum filtration and disinfection for rural community water sustainability, Poster presentation, 2012 IWA Asia Pacific Young Water Professionals Conference, Tokyo, Japan, Dec 7-10, 2012.

Progress Report FY-2012

PI Name: Sangchul Hwang, PhD

Project Number: 2012PR136B

Title: Field Demonstration of Removal of MS2 Bacteriophage and *Bacillus subtilis* with a Solar-Powered Engineered Experimental Drum Filtration and Disinfection (SEED) Unit

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May 16, 2013

Student Training

One graduate and three undergraduate students were hired for the project. For ensuring safety and security, at least two students have always made a team and gone to the field site to get water samples (Photo 1).



Photo 1. Students at the field during the sampling.

The PI (S. Hwang) has trained the students as follows:

1. Propagation and subculturing of *E. coli* and *Bacillus subtilis*,
2. General disinfection theory and chlorine analysis,
3. Explanation of the solar-powered engineered experimental drum filtration and disinfection unit,
4. Measurement of general water quality parameters, pH, TOC, COD, BOD, etc.,
5. Membrane filtration and enumeration of *E. coli* and *B. subtilis*,
6. Set-up and operation of lab-scale sand filters and disinfection reactors,
7. Optical density measurement for *B. subtilis*, and
8. Tracer study to hydraulically characterize the sand filters and disinfection chambers.

The PI and the graduate student, Margaret Hernández attended the WEF-sponsored 2013 Disinfection and Public Health Conference held in Indianapolis, IN on Feb. 24-26, 2013. They also participated in a workshop on the biosand and ceramic filters.

Results Dissemination

Preliminary results were presented at the Asia Pacific Young Water professional Conference, Tokyo, Japan in December 2012:

- Hwang, S., Concepción, D., Hernandez, M. “Solar-powered engineered experimental drum filtration and disinfection for rural community water sustainability.” 2012 IWA Asia Pacific Young Water Professionals Conference, Tokyo, Japan, Dec 7-10, 2012.

Field Source Water Quality Monitoring

Source water for the SEED unit has been monitored for its biochemical characteristics. Tables 1 and 2 show the average values from the duplicate analysis for each water quality parameter.

Table 1. Biochemical quality of the field source water in the first quarter of the project.

Parameter	(unit)	Monitoring dates						
		Mar 27	Apr 18	Apr 24	May 1	May 8	May 16	May 23
pH	-	7.96	8.1	8.3	7.9	7.0	8.1	7.9
Turbidity	(NTU)	0.85	0.75	0.39	0.34	0.64	0.41	0.67
Conductivity	(μ S/cm)	222	222	311	296	251	284	285
COD	(mg/L)	6.1	9.3	5.5	5.5	5.5	- ^a	4.4
Fecal coliform	(#/100 mL)	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
^a not measured								
^b no colony forming unit (CFU) at 10 ² dilution								

Since the second quarter of the project, microbial water quality was monitored for *E. coli* in order to facilitate the project where MS2 bacteriophage will be measured and be tested with the SEED unit. In addition, COD analysis was replaced with total organic carbon (TOC) analysis as the COD values were close to the low detection limit, which might not accurately reflect the actual concentrations of organic matters in the water. Total dissolved solid (TDS) analysis was also included to further support the results of conductivity analysis. Analyses of total coliforms and *B. subtilis* were later included in the third quarter of the project.

As shown in Table 2, all the water quality parameters show a seasonal variation. For example, TDS, *E.coli*, total coli and *B. subtilis* concentration varied 156-241 mg/L, 0-30 CFU/100 mL, 11-1,320 CFU/100 mL and 105-8,900 CFU/100 mL, respectively. This warrants that flexible water purification is needed to accommodate seasonal variation and to produce high quality drinking water.

Table 2. Biochemical quality of the field source water in the second, third and fourth quarters of the project.

Parameter	(unit)	Monitoring dates												
		Aug 28	Sep 6	Sep 13	Sep 25	Oct 9	Oct 16	Oct 25	Oct 29	Nov 12	Dec 4	Dec 18	Jan 22	Jan 29
pH	-	8.5	8.5	8.5	8.6	8.6	8.6	8.7	-	8.7	8.7	8.7	8.7	8.7
Turbidity	(NTU)	0.85	0.51	0.55	0.74	0.57	1.2	0.78	-	0.69	1.11	0.68	1.23	0.72
Conductivity	(μ S/cm)	265	308	333	281	293	247	298	-	281	245	320	325	340
TDS	(mg/L)	156	208	236	200	-	175	212	-	177	174	227	232	241
TOC	(mg/L)	12.5	18.1	19.6	19.6	24.7	2.7	3.5	3.6	1.1	8.3	1.7	0.85	0.3
<i>E. coli</i>	(#/100 mL)	5	6	3	4	11	11	16	3	19	30	1	1	None (10 ⁰)
Total <i>coli</i>	(#/100 mL)	-	-	-	-	TNTC (10 ⁰)	1,050	625	167	710	1,320	21	31	11
<i>B. subtilis</i>	(#/100 mL)	-	-	-	-	TNTC (10 ⁰)	TNTC (10 ⁰)	107	8,900	1,100	900	1,280	105	110

-: not measured
 TNTC: too numerous to count.
 The values in parenthesis are the dilutions at which the microorganisms were measured.

***B. subtilis* Subculturing**

B. subtilis has been maintained by transferring them to the fresh growth medium once every two weeks. The ATCC Nutrient Broth (BD cat 234000) was used as the growth medium. Figure 1 shows the optical density development during the 2-week subculturing.

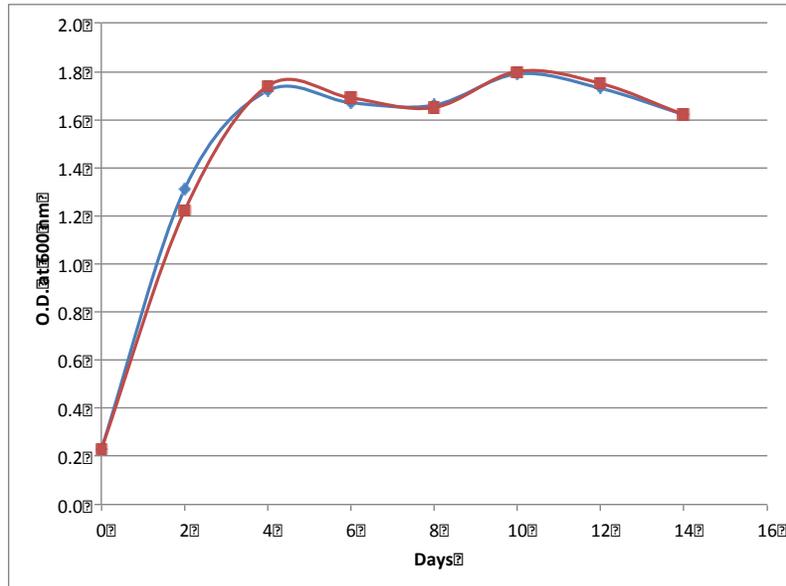


Figure 1. Optical density (at 610 nm) development during the 2-week subculturing. Data are replicated measurements.

Filter Construction

The filters were constructed with a transparent cylindrical acrylic tube (3-in D and 4-in H) (Photo 2). They had a headspace of 1.5 in. Influent was pumped to the bottom. Two different filters were made: sand filter and GAC filter. For sand filtration, two sand filters were connected in series. Table 3 shows the materials and their amount used for construction of the filters. For the GAC filter, the amount of the smallest sand (effective size 0.18 mm) used for the sand filter was replaced by the same amount of the GAC.

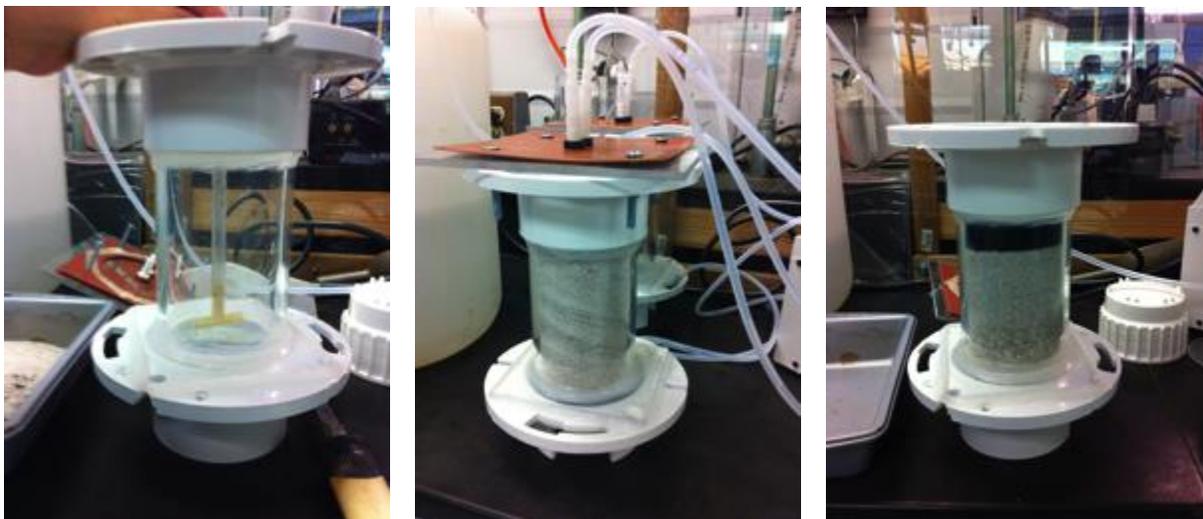


Photo 2. Filter before filtration material packing (left), the sand filter (middle) and the GAC filter (right).

Table 3. The materials used for construction of the filters.

Sand filter:

Media	Size	Total Weight (g)
Sand	Global No. SS 30/65 Effective size=0.18mm	230.2
Sand	Global No. SS 20/30 Effective size=0.55mm	460.4
Sand	Global No. SS 6/20 Effective size=1.10mm	230.2

GAC filter:

Media	Size	Total Weight (g)
GAC	Coal Based CAS No 7440-4-0	230.2
Sand	Global No. SS 20/30 Effective size=0.55mm	460.4
Sand	Global No. SS 6/20 Effective size=1.10mm	230.2

Disinfection Chamber Construction

Transparent acrylic plate was used for construction of the disinfection chambers (Photo 3). The chamber had an effective volume of 3.3 L.

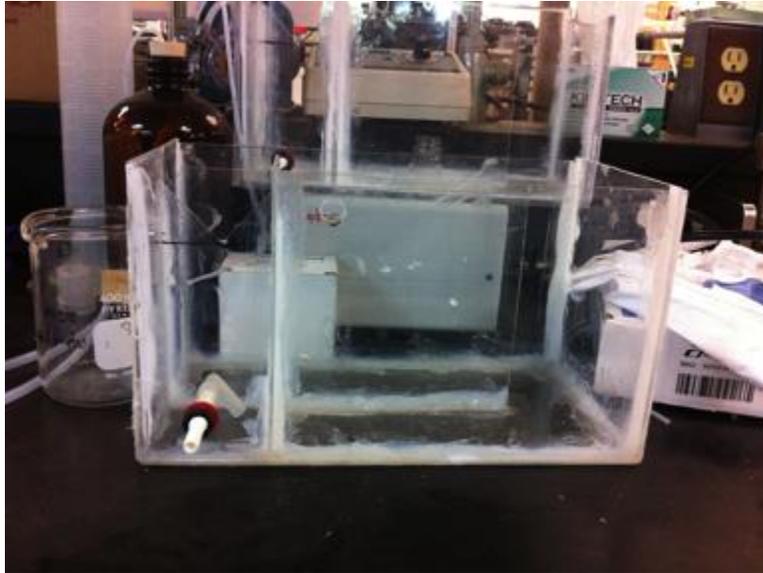


Photo 3. A side view of the disinfection chamber.

Tracer Study

Tracer study was conducted in replicate with the sand and GAC filter to hydraulically characterize the filters (Fig. 2) and to acquire the t_{10} values (Figure 3) that will be utilized for the removal of microorganisms later.

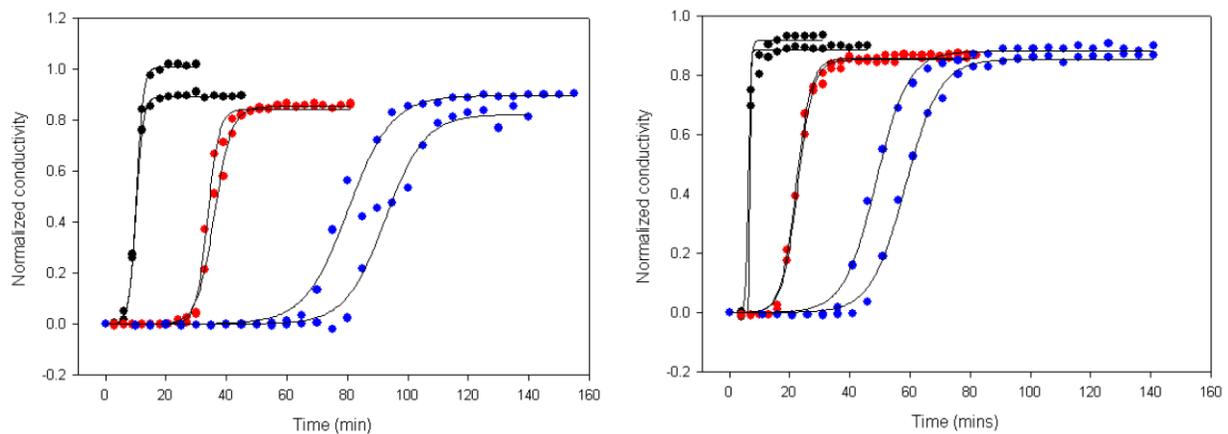


Figure 2. Results of the tracer study with two sand filters in series (left) and the GAC filter (right). Replicated data from the flowrate at 60.6, 11.6, and 1.4 mL/min are shown in black, red, and blue, respectively.

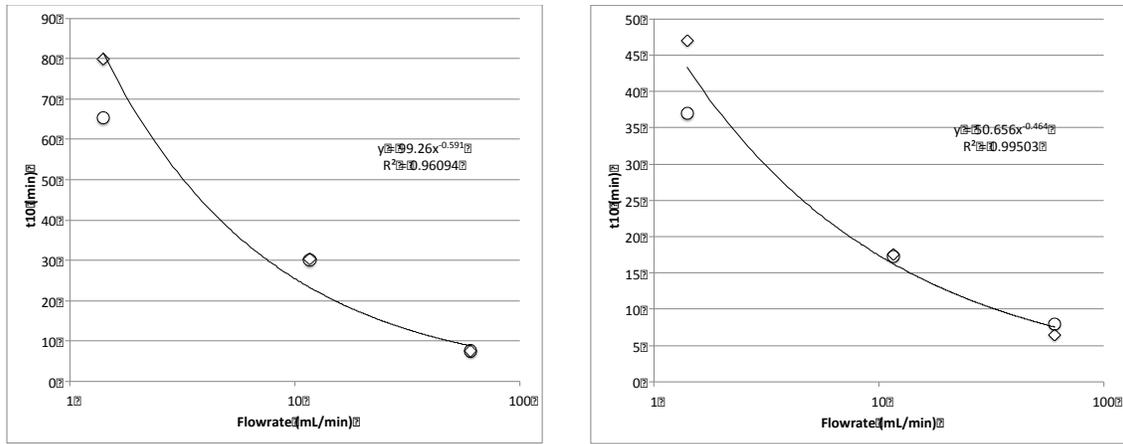


Figure 3. Relationship between t_{10} values and flowrates for two sand filters connected in series (left) and the GAC filter (right).

Tracer study was also performed with the disinfection chamber. Accordingly, the t_{10} value was derived as shown in Figure 4.

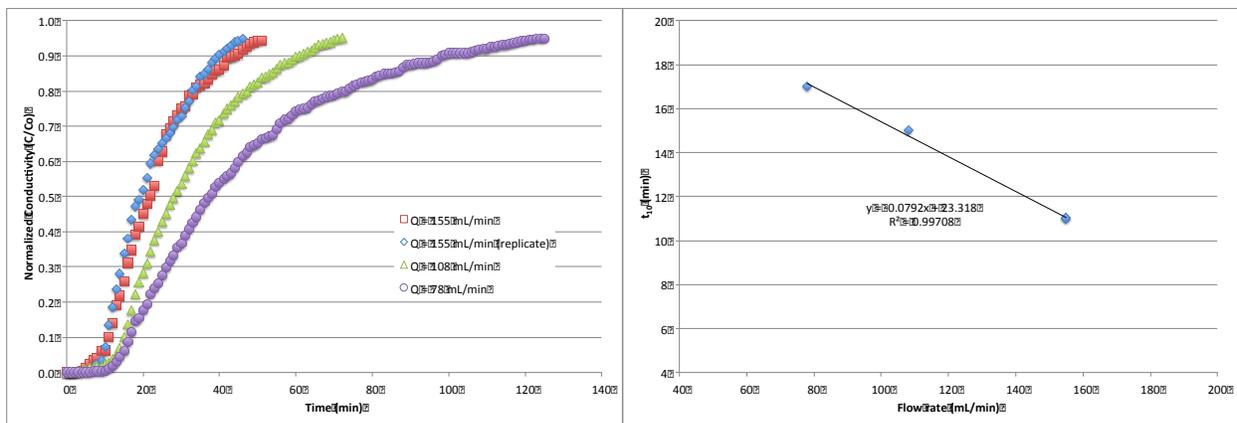


Figure 4. Results of the tracer study with the disinfection chamber (left) and the relationship between t_{10} values and flowrates (right)

On-going Research

- *B. subtilis* removal from disinfection in combination with sand filtration in lab-scale experiment: Log removal of *B. subtilis* will be monitored in a treatment train of sand filters and disinfection chambers (Photo 4).

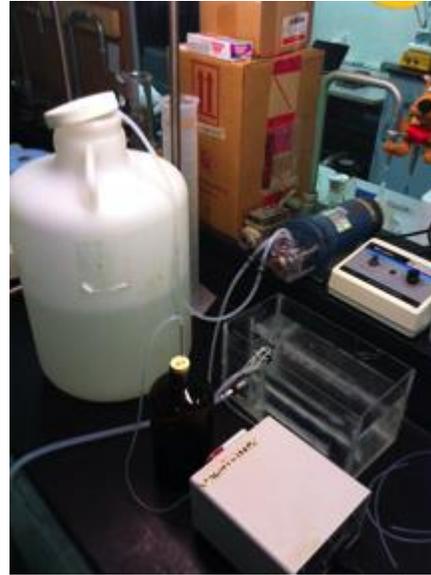
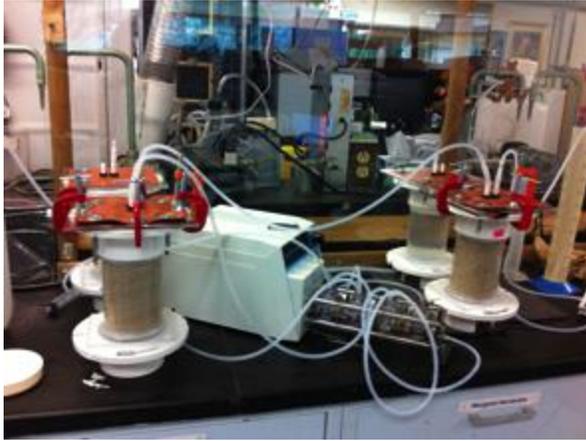


Photo 4. Lab-scale sand filters (left) and disinfection chamber (right).

- Preparation and maintenance of the SEED unit for field-testing: As shown in Photo 5, the SEED unit at field has been physically deteriorated a lot. Therefore, it is necessary to replace those deteriorated parts and to rejuvenate the whole system.



Photo 5. The SEED unit at field (left: a view in ca. 2006, right: a view in 2012).

Acknowledgements

This investigation was supported, in part, by the USGS 104B Program. Administrative support from the Puerto Rico Water Resources and Environmental Research Institute is appreciated.

tracking: The hunt for E. faecalis the dominant Enterococci among non-pigmented environmental enterococci in the water

Microbial source tracking: The hunt for E. faecalis the dominant Enterococci among non-pigmented environmental enterococci in the water systems of Puerto Rico.

Basic Information

Title:	Microbial source tracking: The hunt for E. faecalis the dominant Enterococci among non-pigmented environmental enterococci in the water systems of Puerto Rico.
Project Number:	2012PR138B
Start Date:	4/1/2012
End Date:	2/29/2014
Funding Source:	104B
Congressional District:	
Research Category:	Water Quality
Focus Category:	Recreation, Water Quality, Ecology
Descriptors:	
Principal Investigators:	Luis A Rios-Hernandez, Luis A Rios-Hernandez

Publications

1. Cuebas, M. and Ríos-Hernández, L.A., (2013). The marine environment selects for Rifampicin resistant Enterococci. Oral Presentation, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
2. Magdalis Gonzalez and Luis A. Ríos Hernández (2013). The effect of virulence factors and sugar availability on enterococcal biofilm formation. Oral Presentations, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
3. Valery Lozada and Luis A. Ríos Hernández (2013). Distribution of Antibiotic Resistance Patterns and Vancomycin Resistance Genes in Enterococcus Spp. Isolated from Clinical, Human Fecal, Animal Fecal, River and Beach Samples. Oral Presentations, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
4. Getzabeth Bosque and Luis A. Rios Hernandez (2013). The Dominant Population of Enterococci in Fecal Samples of Healthy Humans is Equipped with the Same Virulence Factors Present in Disease-Causing Enterococcal Strains. Oral Presentations, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
5. Michael Rivera and Luis A. Ríos Hernández (2013). Population Diversity of Enterococcus Spp. in Poultry Fecal Samples. Oral Presentations, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
6. German Rivera and Luis A. Rios Hernandez (2013). Comparison and Dominance of Enterococci Strains Sharing the Same Virulent Genotype from Different Aquatic Environmental Isolates Using a Finger-Printing PCR Technique. Oral Presentations, Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department, Mayaguez, PR.
7. Ginamary Negron and Luis A. Rios Hernandez (2013). Detection of virulence factors among enterococci population from clinical and environmental source in Puerto Rico. Oral Presentations,

Microbial source tracking: The hunt for E. faecalis the dominant Enterococci among non-pigmented environmental

tracking: The hunt for *E. faecalis* the dominant Enterococci among non-pigmented environmental enterococci in the water

- Congreso Internacional de Universidades Promotoras de la Salud y IV Conferencia Puertorriqueña de Salud Pública Centro de Convenciones, San Juan, PR 19 de marzo de 2013
8. Luis A. Ríos Hernández*, Ginamary Negrón Talavera, José A. Romeu, Germán J. Rivera Castellar, Getzabeth E. Bosque Gómez and Michael I. Rivera Morales (2013). False alarms caused by EPA 1600 method in subtropical beach waters. Poster Presentations, U.S. Recreational Water Quality Criteria: A Vision for the Future - held March 11-13, 2013, Honolulu, Hawaii.
 9. Mara Cuebas and Luis A. Ríos Hernández (2013). Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. Poster Presentations, Congreso Internacional de Universidades Promotoras de la Salud y IV Conferencia Puertorriqueña de Salud Pública Centro de Convenciones, San Juan, PR 19 de marzo de 2013
 10. Mara Cuebas and Luis A. Ríos Hernández (2012). The Antibiotic Resistance of *Enterococcus* spp. isolated from Natural Environments, Clinical, Healthy Humans, and Septic Tanks in Puerto Rico. Poster Presentations, Annual Biomedical Research Conference for Minority Students (ABRCMS) San José, CA. November 7-10.
 11. Mara Cuebas and Luis A. Ríos Hernández (2013). Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. Poster Presentations, American Society for Microbiology, May 18-21, 2013 at Denver, CO.
 12. Valery Lozada and Luis A. Ríos Hernández (2013). Distribution of Antibiotic Resistance Patterns and Vancomycin Resistance Genes in *Enterococcus* spp. Isolated from Clinical, Fecal, River, and Beach Samples. Poster Presentations, American Society for Microbiology, May 18-21, 2013 at Denver, CO.
 13. Ginamary Negrón and Luis A. Ríos Hernández (2013). Detection of virulence factors among the populations of environmental Enterococci from recreational marine waters in Mayagüez, Puerto Rico. Poster Presentations, American Society for Microbiology, May 18-21, 2013 at Denver, CO.
 14. Mara Cuebas and Luis A. Ríos Hernández (2013). Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. Poster Presentations, FEM-VI Frontiers in Environmental Microbiology - Mar 15, 2013 Universidad del Turabo, Gurabo, PR.

Annual Report FY 2013

Title: **Microbial Source Tracking: The hunt for *E. faecalis* the dominant Enterococci among non-pigmented environmental Enterococci in the water systems of Puerto Rico.**

PARTICIPANTS

A) What people have worked on your project? In the past year, 2 graduate students (Ginamary Negron y Jose Romeu) and 6 undergraduate students (Getzabeth Bosque, German Rivera, Michael Rivera, Magdalis Gonzalez, Valery Lozada) participated directly in the project.

Name	Role	Time	Task
Getzabeth Bosque	Undergrad researcher	Summer 2012-til now	Goat feces
German Rivera	Undergrad researcher	Summer 2012-til now	Pig feces
Michael Rivera	Undergrad researcher	Summer 2012-til now	Chicken feces
Magdalis Gonzalez	Undergrad researcher	Fall 2012-til now	Bioflim formation
Valery Lozada	Undergrad researcher	Fall 2012-til now	Vancomycin resistance
Mara Cuebas	Undergrad researcher	Fall 2012-til now	Antibiotic resistance
Ginamary Negron	Graduate researcher	Fall 2012- Jan 2013	Beach samples
Jose Romeu	Graduate researcher	Fall 2012- Jan 2013	Horse feces

Name	Gender	Ethnicity	Race	Disability	Citizenship
Luis A. Rios-Hernandez (PI)	Male	Puerto Rican	white	No	US
Getzabeth Bosque	Female	Puerto Rican	white	No	US
Mara Cuebas	Female	Puerto Rican	white	No	US
Magdaliz Gonzalez	Female	Puerto Rican	white	No	US
Valery Lozada	Female	Puerto Rican	white	No	US
Ginamary Negron	Female	Puerto Rican	white	No	US
German Rivera	Male	Puerto Rican	white	No	US
Michael Rivera	Male	Puerto Rican	white	No	US
Jose Romeu	Male	Puerto Rican	white	No	US

B) What other organization have been involved as part of the project? We are collaborating with Donna Ferguson, she is currently a PhD student at UCLA and previously part of Southern California Coastal Water Research Project (SCCWRP). Our collaboration looks at the comparison and frequency of virulence factors found in a diverse Enterococci population from PR and Southern California. We have exchange isolates from animals and beach samples. We are currently writing a scientific article to submit as soon as possible and hopefully publish it this summer. Similarly, we have collaborated with Dr. Marek Kirks form the water Institute and University of Hawaii at Manoa and with Dr. Warish Ahmed from Australia. Both scientists shared either enterococcal DNA or isolates from their regions.

C) Have you had other collaborations or contacts?

During the month of March, I presented a poster at a conference in Hawaii and I was able to meet a few colleagues such as Dr. Alexandria Boehm, Michael Sadowsky, Murulee Byappanahalli, Helena Solo-Gabriele, Valerie Harwood, and Roger Fujioka. These are well renowned scientists from Stanford University, University of Minnesota, USGS great lakes, University of Miami, University of South Florida, and University of Hawaii. This was a great opportunity to meet these scientists, communicate and discuss areas of interests to spark future collaborations.

ACTIVITIES AND FINDINGS

A) DESCRIBE THE MAJOR RESEARCH AND EDUCATIONAL ACTIVITIES.

Research Training

During this past year I had the opportunity to train all of my students (graduate and undergraduates) in the isolation, quantification, and cultivation of Enterococci from different sources including sea water, septic tanks, and animal and human feces. The students perform serial dilutions, membrane filtration, preparation of solutions (PBS buffer), and learned how to prepare liquid and solid media. They were able to apply aseptic techniques and store all the isolates in glycerol stocks for future usage.

Another area in which the students have acquired considerable experience through this grant is in molecular biology. In this area the students have learned how to extract DNA from pure cultures; to do PCR amplifications, develop new PCR protocols, design species specific PCR primers (blast search, sequence alignments), and troubleshoot PCR; also to run agarose gels, polyacrylamide gels, and interpret their results using all of those techniques.

The most important activity, as I see it, is the exercise all of the students participating in this research grant go thru when they have to start up an experiment for the first time. They must present to me their experimental design and their hypothesis they are testing in their experiments. In this experience the students learn firsthand one on one with me how to formulate a hypothesis, how to test the hypothesis designing the right experiments, and which controls need to be used to be able to accept or reject their hypothesis.

Educational activities

Laboratory meetings are held every week (Wednesday at 6pm), it is the time in which we discuss the events that happen during the previous week and solve all the problems, organize the materials that need to be purchased, etc. More importantly, it's a great opportunity to teach all the students communication skills by assigning one student to present their research to the lab mates. I have to confess that this is being more difficult than I anticipated. I expect to see a well presented power point presentation, both visually (without grammar and spelling errors) as well as organized. We also discuss what to avoid during the presentation (mannerisms, jargon, posture, etc).

Furthermore, the participating students must write a preliminary laboratory report (two months into the semester) and a final laboratory report (end of semester) both in English, as well as keeping a laboratory notebook throughout their career in our laboratory. These practices allow me to teach the participants writing skills, responsibility, organization, adequate data collection and management, as well as polish their scientific writing skills which it turns out to be the most frustrating and difficult aspect of the whole project.

This past week, on May 4th, all of the undergraduate students presented their research projects in our 3rd undergraduate research symposium at the biology building. This was a great opportunity to disseminate scientific information and to do a self-evaluation of their own work and how does it compare to other undergraduate researchers. I am proud for all their efforts (6 presentations from my lab out of 89 presentations in all). I am especially proud that two of my students were awarded with best talk in their session.

This coming month of May I will travel to Denver, CO, to participate in the American Society for Microbiology annual meeting. Fortunately, I will go with two undergraduate students and one graduate student to present their work in three different posters related to our research project. The two undergraduate students will participate in a scientific meeting of this size (8,000 people) for the first time in their scientific careers. I believe this will be the best educational-research training activity with the fastest turn around effect. In my experience, this activity usually results in a great experience for them and stimulates their desire to continue in a scientific career. I am sure that these students will never forget this experience. We were fortunate to apply for a travel grant for students and all of them were selected to participate since we did not have enough funds to cover their travel expenses.

B) DESCRIBE MAJOR FINDINGS RESULTING FROM THE ACTIVITIES.

The current regulations to assess recreational waters (marine) dictate that the presence of enterococci at levels higher than 104 CFU/100ml in a single sample or 35 CFU/100ml in geometric mean (5 consecutive samples within a month) deems the beach unsafe for swimmers. It actually assumes that all the enterococci enumerated were originated from fecal matter. In fact we have shown that in Puerto Rico, and others in tropical environments, this is not true. In Puerto Rico, and possibly other places, a motile and yellow pigmented enterococci that it is not typically associated with humans is the dominant enterococci. This organism has been identified as *E. casseliflavus*, using both phenotypic characteristics as well as molecular analysis of its genetic material.

The less dominant members of the enterococci community commonly isolated from our recreational waters belong to the non-pigmented non-motile group typically represented by *E. faecalis* and to a lesser extent *E. faecium*. In our previous report, we were able to differentiate between individuals of the same species across the “recent contamination event” by using a multiplex PCR that amplified 5 different virulent genes. To our surprise, our samples were dominated by *E. faecalis* with a virulent genotype (*gelE/asa1*). In this project, we explored the natural reservoir of this group of organisms which we believe that this genotype contributes to its persistence, prevalence, survival, dominance, and overall success in the water systems of Puerto Rico. Finding the

reservoir of the *E. faecalis* with this genotype will allow us to identify the potential source of contamination and determine if it actually comes from human fecal matter or if its distribution is more ample. Furthermore, understanding the physiological and ecological advantages that these isolates acquire will allow us to determine their potential niche within this natural environment.

MATERIALS AND METHODS

1. The enumeration, characterization, and speciation of enterococci from water and fecal samples.

Sample collection: The seawater samples were collected previously (see last year's report) in the west coast of the Island of Puerto Rico in the municipality of Mayaguez and Añasco. The isolates from last year's project were preserved in glycerol stocks and used for this year's analysis. New samples were collected from septic tanks, fecal samples from animals and healthy human volunteers. Clinical samples were donated from a local laboratory within a local hospital in Puerto Rico. These samples were purified and identified by licensed personnel of the hospital; the individual isolates were received on streaked onto a blood agar plate with a list that contain the isolate identification number, species, and site of the body that was collected from, absolutely no information what so ever from the patient or outpatient was obtained from the hospital.

Animal and healthy human fecal samples: We collected samples of feces of different animals from a nearby farm including: chickens, pigs, goats, and a horse. From each individual group of animals we collected a fecal sample from fresh droppings or directly from the animal. The samples were pooled in a sterile whirl pack plastic bag and homogenized before further analysis. For the isolation of colonies from the feces, we weight approximately 1.00g of the composite sample and transferred it to a bottle with 99 mL of PBS (50 mM, pH 7.4) (previously sterilized), and then proceeded to homogenize the sample vigorously by hand for approximately 1 minute until all the solid was diluted in the buffer. Then a series of dilutions from 10^{-3} to 10^{-7} were made and filtered through a 0.45 μm membrane and cultivated on mE medium for 48 hours at 41°C. Once the 48 hours passed, the colonies were counted and 50 individual colonies were randomly chosen, and transferred to Brain Heart Infusion Broth. The isolates were incubated for at least 18 hours at 37°C. Every colony had a different ID number/name to avoid further confusion and ease cataloging. To confirm the purity of the isolates we performed the four quadrant streak plate on Brain Heart Infusion Agar. After pure colonies were observed, a single colony was transferred to a new plate to create biomass to preserve as glycerol stocks. Each isolate from mE was considered a presumptive Enterococci and a series of biochemical tests were done to be sure that the samples were in fact an enterococci. The healthy human isolates were obtained similarly; they were collected by the volunteer donors in pre-sterilized commercially available sampling cups purchased at a local drug store. These samples were kept separately as individual samples unlike the animal samples that were a composite. The septic tank samples were collected in two ways; some of the samples were collected directly from the septic tanks by owner's volunteers that remained anonymous and by collecting from trucks that clean septic tanks. The collection was done directly from the truck tank prior to emptying, usually the trucks contained material from one or two different houses. Each individual samples, per

trucks were kept and analyzed individually. Once the samples were in the laboratory they were analyzed as described above.

Biochemical tests: A series of biochemical tests were done to ensure that the isolates belong to the genus *Enterococcus*. Pigmentation was done by collecting biomass from an overnight grown culture on BHI with a sterile white cotton swab. Any yellowish pigmentation, from intense to light was considered as pigmented. The same swab with the sample was used to test for catalase using a 3% commercial OTC hydrogen peroxide solution. The positive control for the catalase test was *S. epidermidis* isolated from the skin of a volunteer. To test for motility we inoculated with a needle every isolate into Sulfide-Indole-Motility medium (SIM). After at least 18hrs of incubation at 35°C, the samples were compared to a positive control (*E. casseliflavus*) and a negative control (*E. faecalis*). The isolates were also tested for their ability to grow at high concentrations of salts (BHI 6.5% NaCl), their ability to grow at 45°C on BHI, and the ability to hydrolyze esculin using the Bile Esculin Agar medium (BEA). All the isolates that were confirmed as Enterococci were subjected to further characterization using molecular techniques.

Antibiotic resistance test. The isolates used in the biochemical test were also tested for their resistance to different antibiotics. We used the Kirby-Bauer Method to cultivate the isolates in Muller Hilton Agar (Bauer *et al.*, 1966). The Petri dish was divided in four quadrants and each quadrant had a different antibiotic. The antibiotics that we used are: vancomycin (30µg), piperacillin (100µg), tetracycline (30µg), and rifampicin (5µg). After 24 hours of incubation at 37°C, the diameter of inhibition was measure with a ruler and then classified as resistant, intermediate or susceptible based on the manufactures recommendations.

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 95°C 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. Alternatively, the DNA was extracted using a protein method especially for the Box-PCR method (Jackson, 2112).

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). Verification of any species was done using the multiplex PCR method described by Jackson (2004).

2. To detect the presence of virulence factors among the population of 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). The thermal cycler starts with at 95°C for 15 minutes followed by 30 cycles of denaturation (94°C for 1 minute), annealing (56°C for 1 minute), and extension (72°C for 1 minute), followed by one cycle consisting of 10 minutes at 72°C. 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).

Vancomycin resistance gene PCR. The isolates with resistant or intermediary phenotype for vancomycin using the Kirby-Bauer antibiotic test were then analyzed by a Multiplex PCR assay for *vanA*, *vanB*, *vanC1*, and *vanC2/3* previously described (Bell 1998). PCR amplification in 50µL reaction mixtures containing dNTPs at a concentration of 200µM, each primer at a concentration of 1 µM, MgCl₂ at a

concentration of 25mM, and 1U of Taq polymerase in Green Buffer. The samples were subjected to 35 PCR cycles using MyCycler Thermal Cycler from Bio-Rad. Each one of these cycles consisted of 1 minute of denaturation at 94°C, 2 minutes of annealing at 60°C, and 2 minutes of elongation at 72°C. After that PCRs were analyzed by electrophoresis (100v for 75min) on 1.8% agarose gels (stained with etidium bromide). The oligonucleotide primers used were vanABF with sequence GTAGGCTGCGATATTCAAAGC and specific for vanA and vanB gene, vanAR with sequence CGATTCAATTGCGTAGTCCAA and specific for the vanA gene, vanBR with sequence GCCGACAATCAAATCATCCTC and specific for the vanB gene, vanC1F with sequence TGGTATTGGTATCAAGGAAACC and specific for vanC1, vanC1R with sequence AGATTGGAGCGCTGTTTTGTC and specific for vanC1, vanC23F with sequence CAGCAGCCATTGGCGTACAA and specific for vanC2 or vanC3, and vanC23R with sequence CAAGCAGTTTTTGTAGTAGTTC and specific for vanC2 or vanC3. For each sample, two PCRs were made. The first one contained the primers VanABF, VanAR and VanBR, which amplified two genes: vanA of 231-bp and vanB of 330-bp. The second PCR contained the primers VanC1F, VanC1R, VanC23F and VanC23R, which also amplified two genes: vanC1 of 447-bp and vanC2 or vanC3 of 597-bp. Known positive control for vanA, and a negative control were also included in each PCR run.

3. To determine the strain similarity among the population of Enterococci.

Box-PCR method: The previously extracted DNA using the protein method was used in this PCR protocol. The DNA was quantified using a Nanodrop and all of the samples were adjusted to 50ng/ul using TE buffer prior to the actual amplification. The master mix consisted of 9.5µL ddH₂O, 5.0µL of 10x Green GoTaq (Promega), 3.5µL MgCl₂ (25mM), 2.5µL of dNTP's (2.5mM), 0.25µL 100x Purified BSA (New England BioLabs), 2.0µL BOX1R primer (20pmol) and 0.25µL of 5u/µL taq polymerase GoTaq Flexi (Promega) for a total of 23µL plus 2µL of DNA 50ng for each sample. The product of BOX PCR was run on a 2% agarose gel for 4 and half hours at 60V stained with ethidium bromide, and visualized by UV using a molecular imager system (BioRad VersaDoc MP 4000).

RESULTS

Last year's new developments and the topic of this year's proposal were summarized like this: "We have discovered that in Puerto Rico the dominant enterococci in seawater are pigmented and motile. Among the pigmented species found by our previous work, *E. casseliflavus* is the most abundant followed by *E. mundtii* and *E. sulfureus*. Among the non-pigmented, *E. faecalis* is dominant within parameters while *E. faecium* dominates when the samples are out of parameters. More importantly, we were able to distinguish between two strains of *E. faecalis* that are commonly found in seawater samples based on their genotype by using the presence of five genes among their genome (**see preliminary data**). Using this molecular technique we were able to observe the introduction of new genotypes into the seawater system. The source of these enterococci is currently unknown, but their importance is exacerbated by the fact that

enterococci isolated in Southern California (collaboration with Donna Ferguson) also possess the same genotypes as the isolates we find in PR.”

In this proposal we determine the distribution of this particular Enterococci that dominates in Puerto Rico’s waterways and show evidence that these organisms are shared between; the beach samples across continents, healthy human and animal feces, septic tanks and even clinical samples. The common factor that all these organisms share and makes them successful in all these different environments could be their virulent genotype of *gelE/asa1*. The physiological advantage that these genes confer to *E. faecalis* is explored but our results might be limited to have final conclusions.

Distribution and range of *E. faecalis* isolates with the same virulent genotype: In Figures 1 and 2 we show evidence that these particular Enterococci are distributed around the world. We were able to show that these organisms could be isolated not only in PR’s beaches, waterways, and clinical samples, but also they are present in the Ohio River, Southern California, Hawaii, and Australia.

Clinical and environmental isolates with the virulent genotype from Puerto Rico

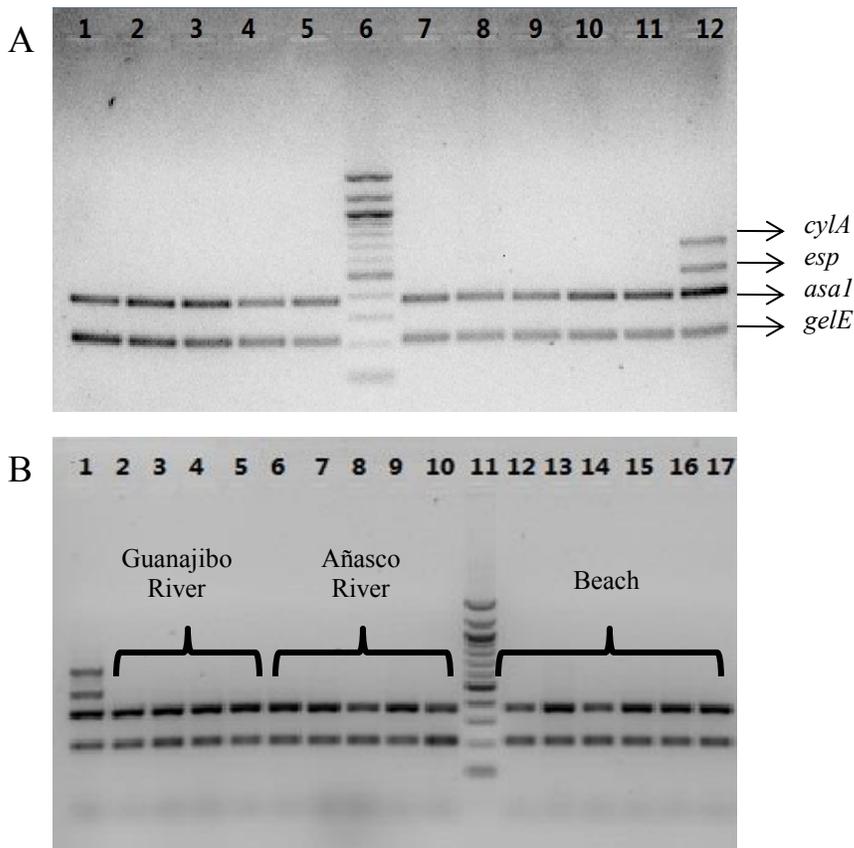


Figure 1. The dominant virulent genotype among Enterococcal isolates from A) clinical isolates and B) from different water samples. Positive controls A1 and B1. Negative controls not shown.

Environmental isolates from diverse
worldwide with the same virulent genotype

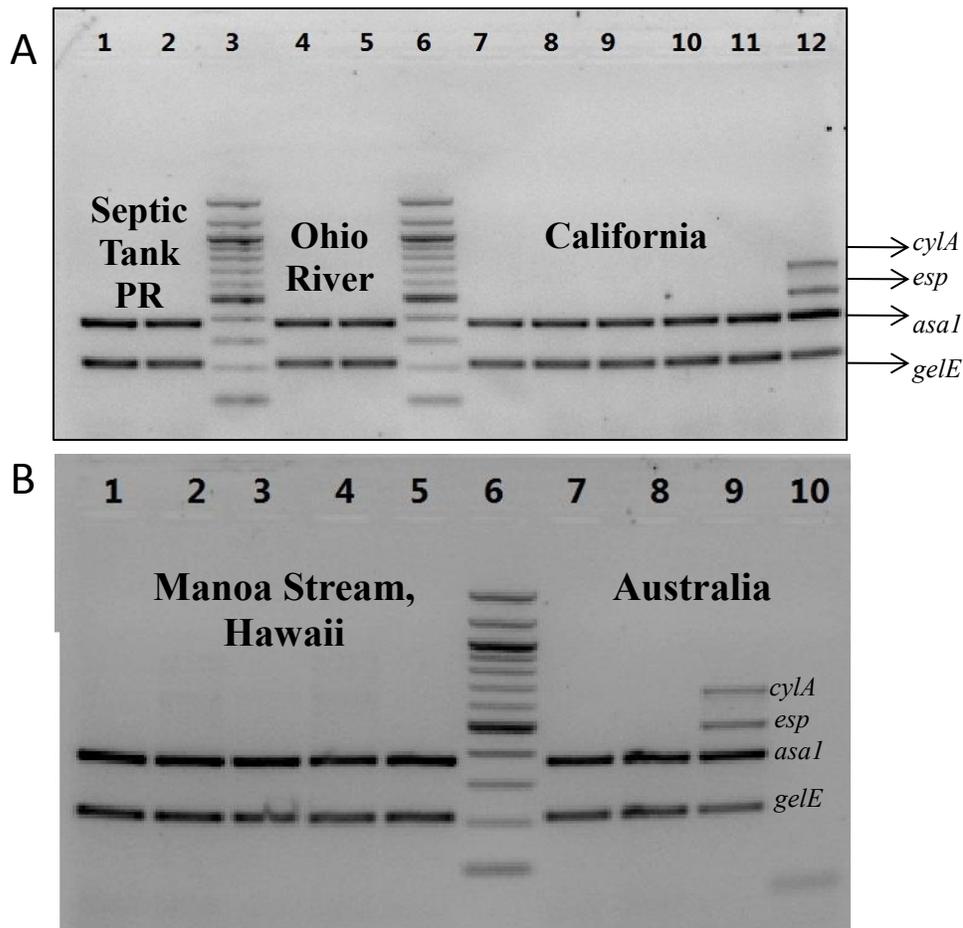


Figure 2. A. Enterococci with the same virulent genotype from septic tanks in Puerto Rico, Ohio river isolates, and in birds from California. B. Isolates from Hawaii and Australia also contain the same virulent genotype. Positive controls A12, B9, and negative control B10.

Interestingly, we also collected evidence that shows that these organisms can also be isolated from healthy human fecal matter. In Figures 3 and 4 we observe individuals that are carriers of these Enterococci. Also we show evidence that the human GI tract could be dominated by one Enterococci with a particular virulent genotype, or co-dominated by two different species of Enterococci with different virulent genotypes (see Figure 3). Furthermore, we show that different non-related humans could share the same Enterococci population as seeing in the comparison of Figures 3 and 4. Notice that both individuals, male and female, were populated by *E. faecalis* and/or *E. faecium*, and that *E. faecium* did not contain any virulent genes. It is important to differentiate that Figure 3 represent two samples of the same individual at two different time, while Figure 4 represent one sample, a snap shot of the Enterococci population at that particular time.

Speciation and virulence factors in fecal isolates from a healthy human.

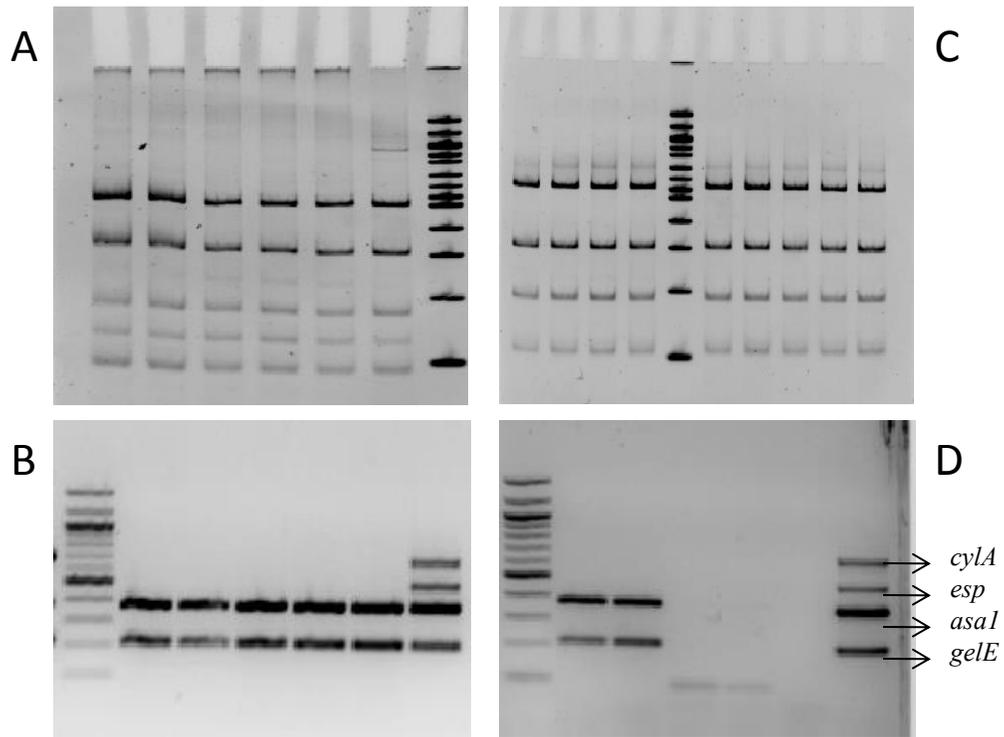


Figure 3. A. RFLP demonstrating that all isolates belong to the same species (*E. faecalis*) in M43 samples. B. All isolates from M43 have the same virulent genotype. C. RFLP demonstrating that all isolates belong to the same species (*E. faecium*) in M43B samples. D. Virulence factors present in fecal sample from healthy human M43B. Notice the transition of species and virulence factors from M43 to M43B, it is the same individual but at different sampling times.

Speciation and virulence factors in fecal isolates from a healthy human.

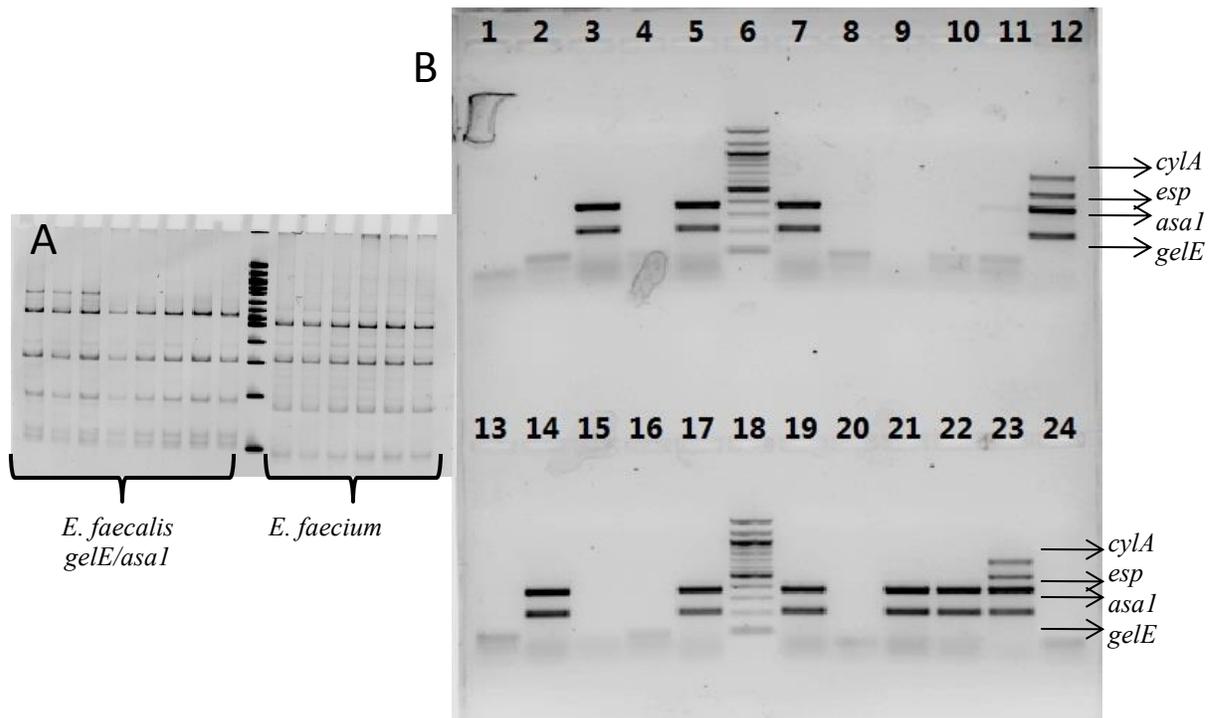


Figure 4. A. RFLP of the α -sub unit of atp synthase to speciate the isolates from healthy human F19. Virulence factors in isolates from F19. The genotype *gelE/asa1* is limited to *E. faecalis* isolates exclusively while *E. faecium* does not have any virulence factors.

Furthermore, Figure 5 shows evidence that the enterococcal population within healthy humans varies through time both in species composition and the virulent genotype that they possess. In this particular example we observed, within the same individual, the transition/substitution of two different species of Enterococci from M25 to M25B without a virulent genotype to a mixture of virulent genotypes dominated by *gelE*. More importantly, the virulent genotype *asa1/gelE* is also present in M25B, D panel lane 3 top.

Virulence factors in fecal isolates from a healthy human.

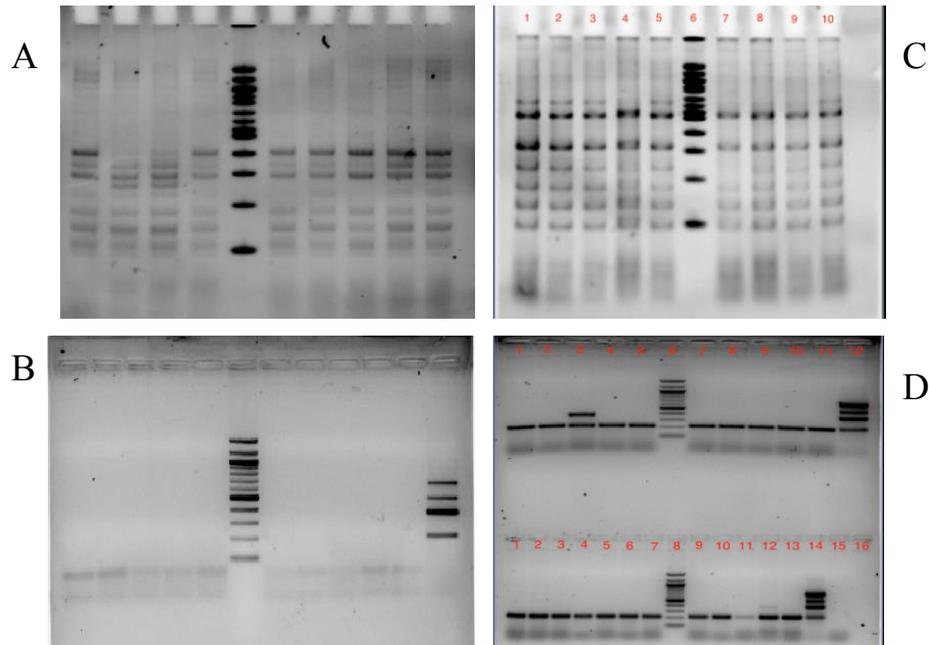


Figure 5. A. RFLP demonstrating that all isolates belong to the same species in M25 samples. B. Absence of virulence factors in isolates from healthy human M25. C. RFLP demonstrating that all isolates belong to the same species in M25B samples. D. Virulence factors present in fecal sample from healthy human M25B. Notice the transition of species and virulence factors from M25 to M25B, it is the same individual but at different sampling times.

Furthermore, we show evidence of two individuals, not related, a male and a female, that contain the same dominant species (*E. faecalis*) and all of their isolates contain three virulence factors (Figure, 6). It is also important to point out that we found healthy humans that have Enterococci with four of the five virulent genes we were interested in (Figure, 7) as well as other individuals that their fecal Enterococci do not have any virulent factors yet all of them appear to be healthy individuals.

Speciation and virulence factors in fecal isolates from healthy humans M39 and F26.

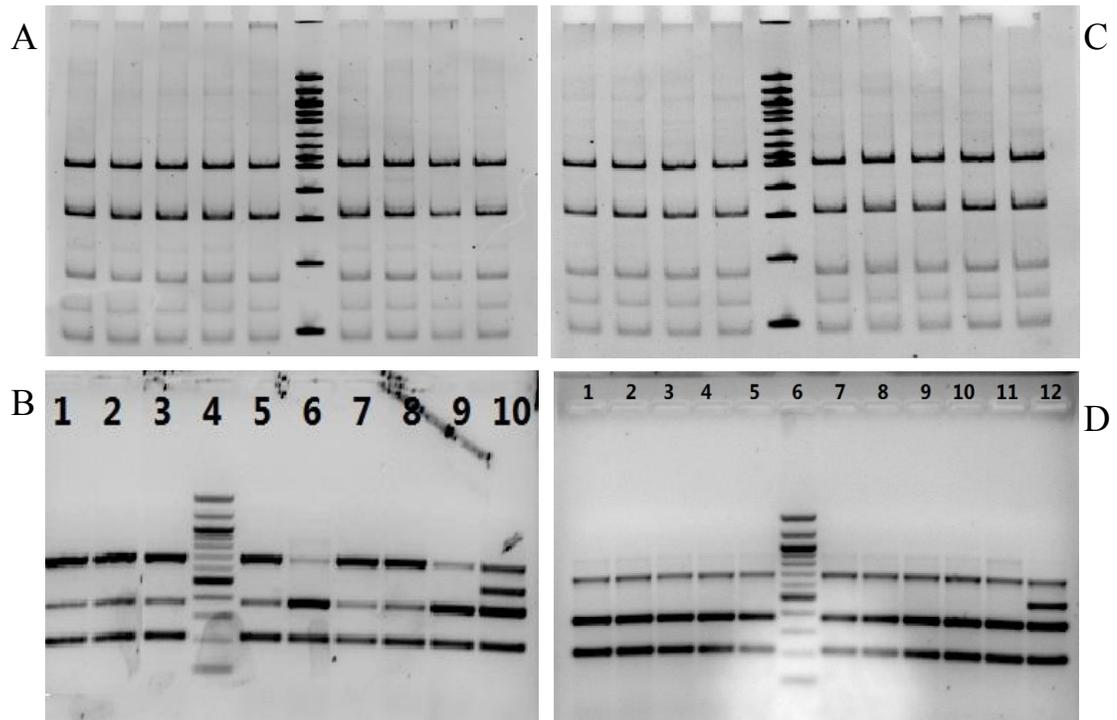


Figure 6. A. RFLP demonstrating that all isolates belong to the same species in M39 samples. B. Virulence factors in isolates from healthy human M239. C. RFLP demonstrating that all isolates belong to the same species in F26 samples. D. Virulence factors present in fecal sample from healthy human F26. Notice that the same species (*E. faecalis*) is dominant in both samples. Furthermore, the isolates also share the same virulence factors.

Speciation and virulence factors in fecal isolates from healthy humans.

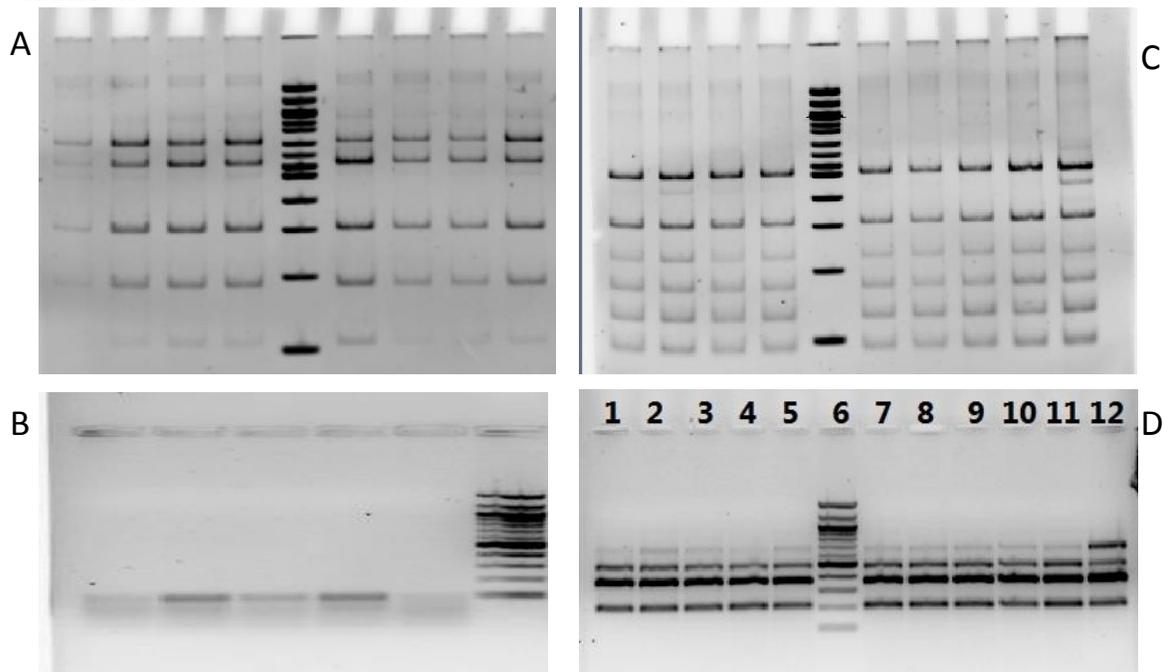


Figure 7. A. RFLP demonstrating that all isolates belong to the same species in M68 samples. B. Absence of virulence factors in all isolates from healthy human M68. C. RFLP demonstrating that all isolates belong to the same species in M20 samples. D. Virulence factors present in fecal sample from healthy human M20. A and C are inverted ethidium bromide stained polyacrylamide gels. B and D are inverted ethidium bromide stained agarose gels. Positive control lane 12D.

As part of our efforts to define the origin and range of the dominant non-pigmented Enterococci found in beach samples around the world, we looked into animal feces as a possible reservoir. Interestingly, as shown in Figure 8, we were unable to find the virulent genotype (*asa1/geIE*) in any of the isolates obtained from pigs. Basically the majority of the isolates contained only one virulent factor, *geIE*. Likewise, in Figure 9, we show that the isolates from goats do not contain the virulent genotype that we were looking for and it is relevant in the marine samples. We do amplify a non-specific band at 1.5kb in the majority of these isolates. We do not know at this time what this amplification product is but it seem to be dominant among goats and perhaps this isolate is shared with pigs (Figure 8, lane 4 bottom).

Virulence factors in Enterococci isolates from pig feces

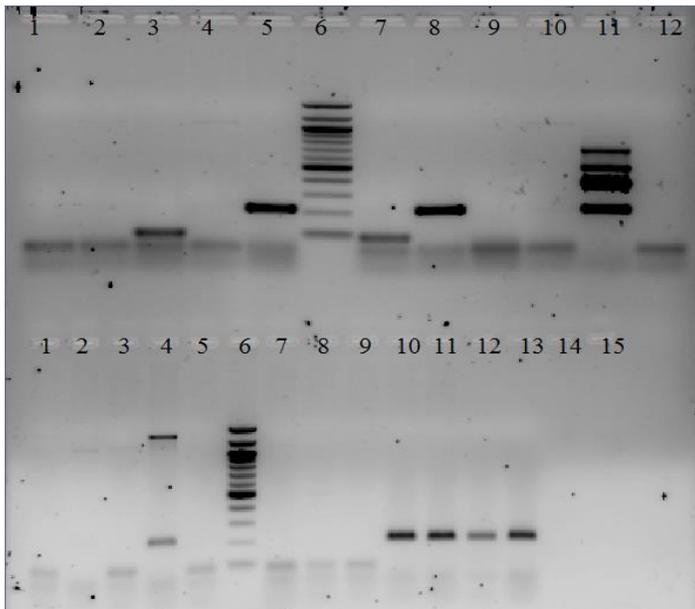


Figure 8. The virulent genotype among Enterococcal isolates from a composite fecal sample from pigs. Positive control on top lane 11, negative control on top lane 12. Inverted ethidium bromide stained agarose gel.

Virulence factors in Enterococci isolates from goat

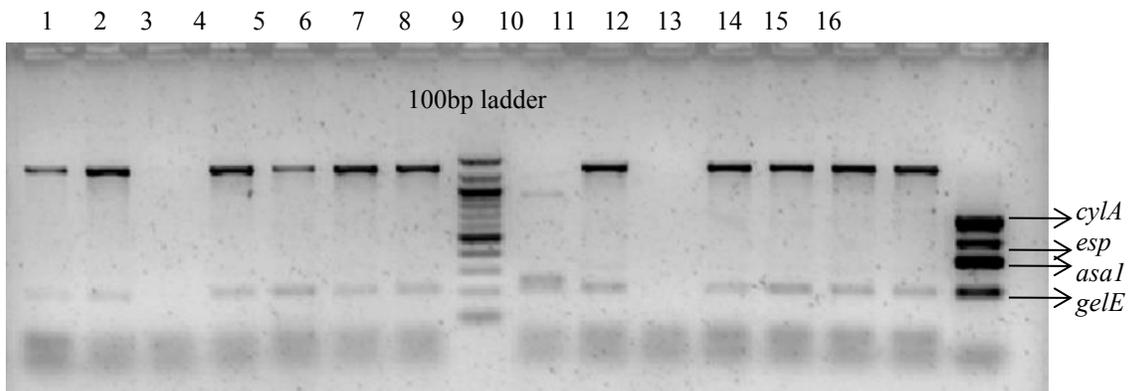


Figure 9. The dominant virulent genotype among Enterococcal isolates from a composite of goat fecal sample. Positive control on lane 16, negative control not shown. Inverted ethidium bromide stained agarose gel.

Another animal that was assayed was horses. We found that the population of Enterococci dominating their fecal matter was *E. casseliflavus*, a yellow pigmented and motile organism that does not contain virulence factors (data not shown). Finally, we analyzed chickens (shown in Figure 10) as a possible reservoir and found that the dominant virulence genotype among these isolates was *asaI/gelE*. Surprisingly in these

Virulence factors in Enterococci isolates from chicken feces

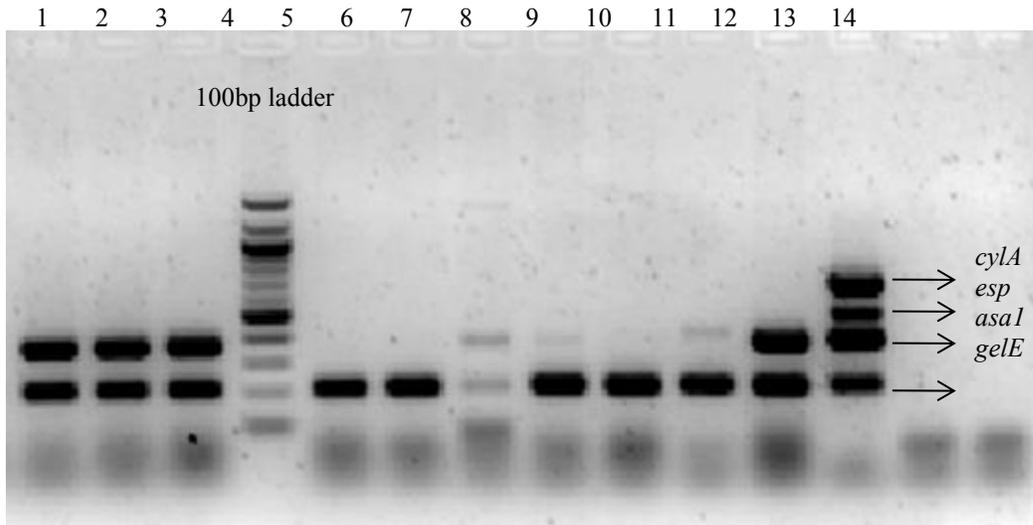


Figure 10. The dominant virulent genotype among Enterococcal isolates from a composite fecal sample. Positive control lane 12, negative control lane 13 and 14. Inverted ethidium bromide stained agarose gel.

animals we found the highest frequency of isolates with the virulent genotype of interest, but as seen before these organisms appear to be present/shared between some humans, natural habitats, septic tanks, and clinical samples. Furthermore, they seem to be absent among pigs, horses, some humans, and goats.

Comparison of *E. faecalis* isolates with the same virulent genotype using BOX-PCR as a fingerprint technique: In figure 11, we show that it appears that the dominant Enterococci in human feces is limited by a single strain per individual but it is highly diverse among all humans. We also witness different individuals that showed co-existence of more than one enterococcal strain within the same sample (M39, F19, M43B, and C) and even some strains that are common in different individuals without an apparent relationship among the individuals (F19, M43B, and C).

Strain diversity of Enterococci isolates from Healthy Human

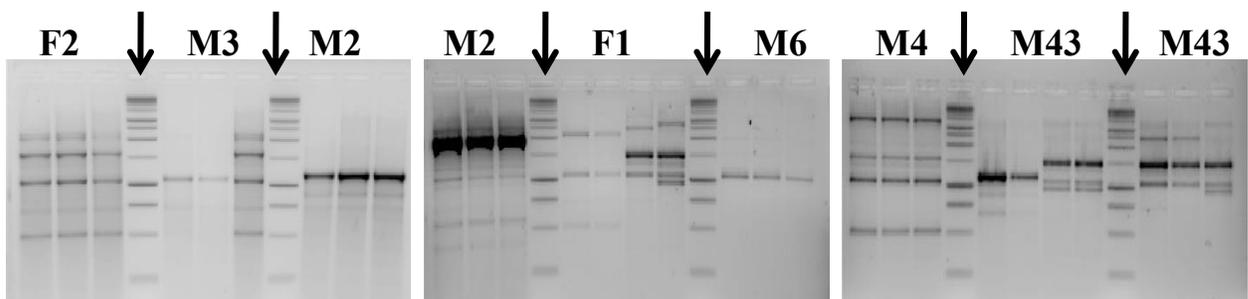


Figure 11. Box PCR describing the diversity and similarities of isolated strains from healthy Humans. We show the representative dominant strains of 7 individuals. M43, M43B, and M43C show strain transition through time. Arrows denote 1Kb ladders. Inverted ethidium bromide stained agarose gel.

Unlike humans, the animal enterococci population seems to be more diverse in species and strains of the same species. In Figure 12, we can observe the diversity of strains within the population of Enterococci in pig feces. Interestingly, we can observe at least

Strain diversity of Enterococci isolates from pig feces.

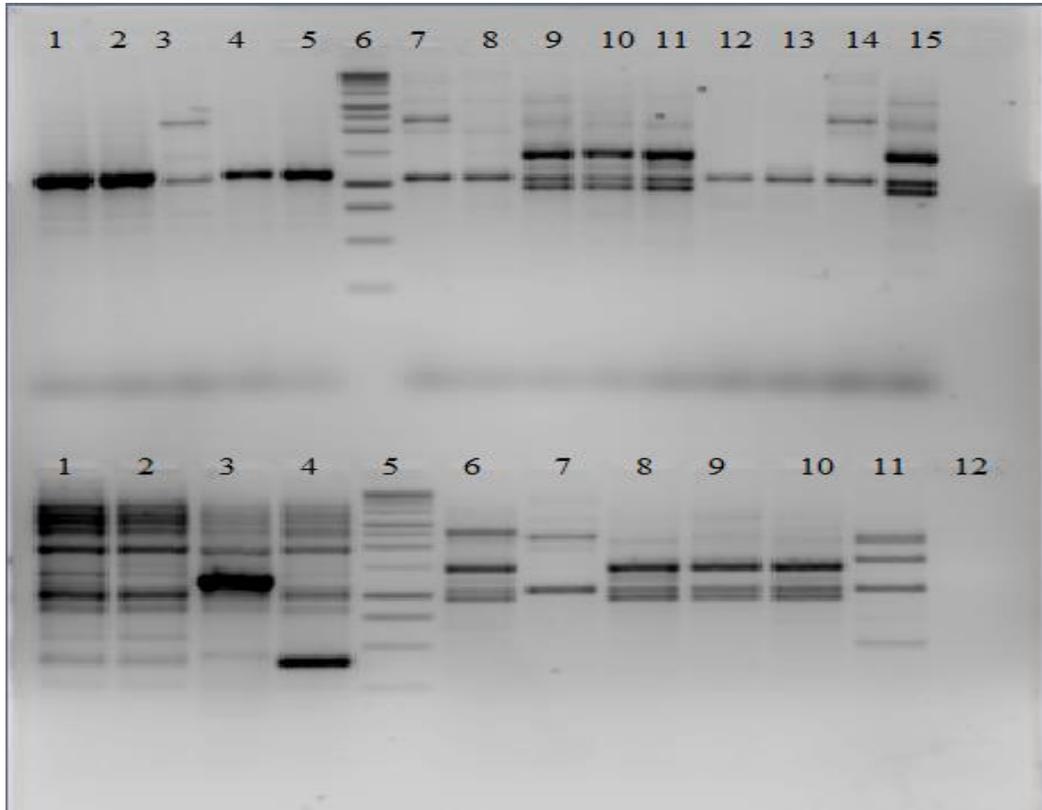


Figure 12. Box PCR describing the diversity and similarities of isolated strains from pig feces. Inverted ethidium bromide stained agarose gel. Positive control on lane 11 on bottom. Inverted ethidium bromide stained agarose gel.

eight different strains in 23 isolates. Similarly, in the feces of chickens we can see a diverse group of enterococci strains (Figure, 13). In 22 isolates analyzed we can see at least eight different strains but one strain seems to be dominant with a higher frequency as exemplify by lanes 5 to 14 in the bottom lanes.

Strain diversity of Enterococci isolates from chicken feces.

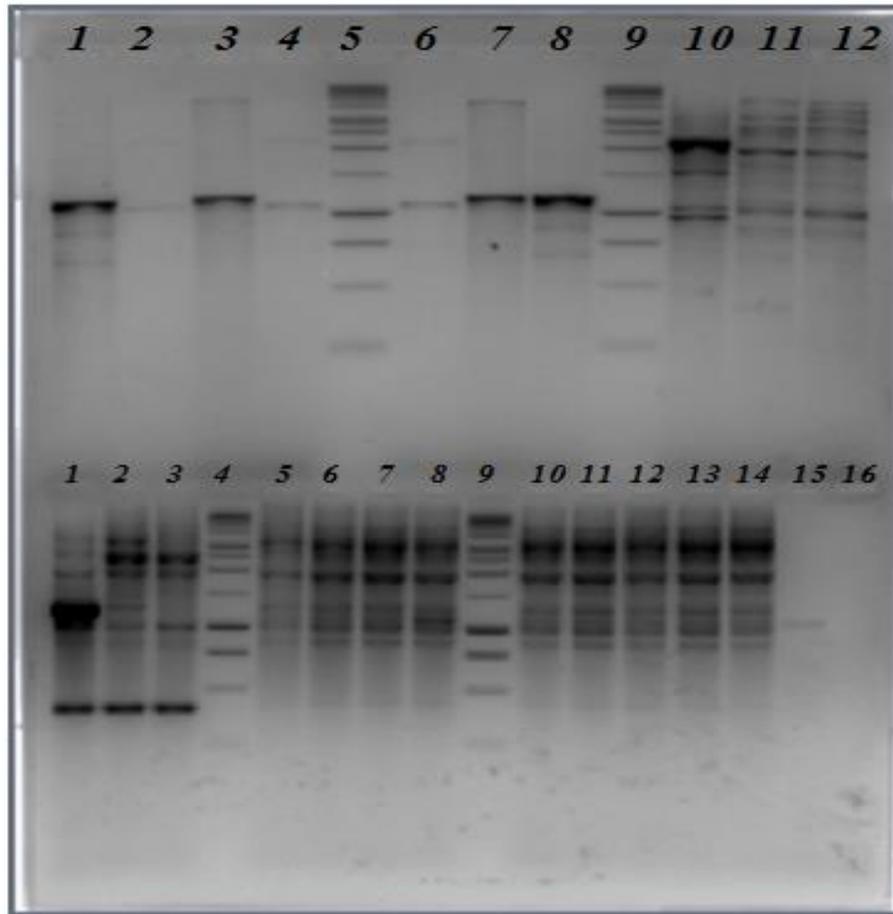


Figure 13. Box PCR describing the diversity and similarities of isolated strains from chicken feces. Inverted ethidium bromide stained agarose gel.

In comparison, when the beach isolates were analyzed we observe a highly diverse group of strains even within the same time interval (Figure, 14). Interestingly the marine environment seems to be dominated by different strains at each 4 hour interval. There are strains that seem to be isolated more frequently than others throughout the 24 hour period (12pm, 8pm, and 12amII).

Strain diversity of Enterococci isolates from marine water samples collected at 4 hour intervals for 24 hours.

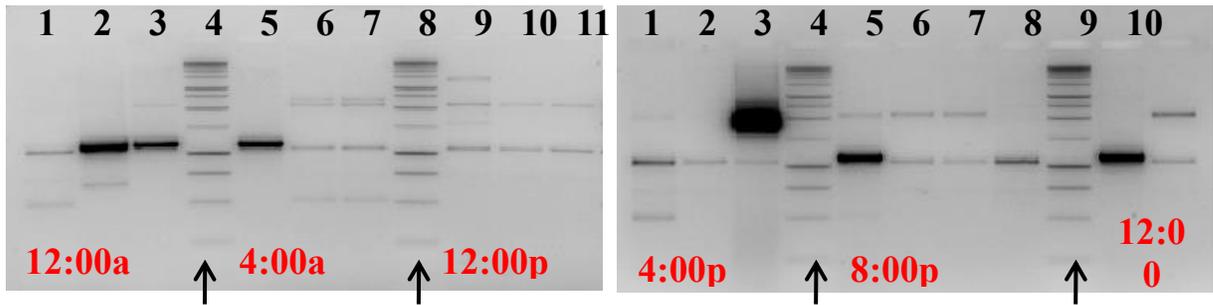


Figure 14. Box PCR describing the diversity and similarities of isolated strains from marine water samples. We show the representative dominant strains of 6 time interval samples at the same location . Arrows denote 1Kb ladders. Inverted ethidium bromide stained agarose gel.

In order to determine if these isolates and strains are shared between different locations we constructed Figure 15. Here we compare the most similar strains from each source to determine the range of the isolates. We found that strains are shared between humans and pigs panel B M39 and panel C M43B, to our surprise we could not demonstrate that the beach samples shared any strains with any of the other samples, human or animal.

Comparison of Enterococcal strains isolated from marine water, birds, pigs,

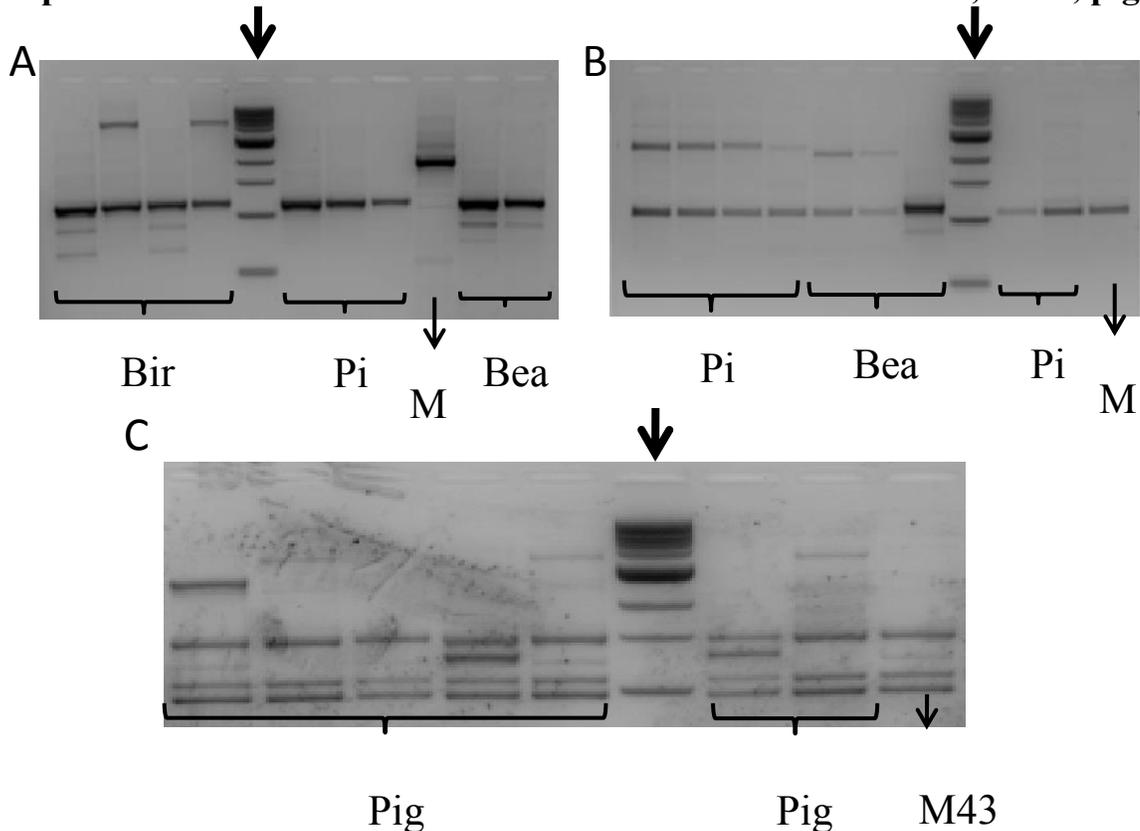


Figure 15. Box PCR describing the diversity and similarities of strains of Enterococci isolated from animals, beach, and healthy humans using the primer BOXAR1. We show the representative from each group that shows the most similarity to other isolates from different groups. Arrows denote 1Kb ladders. Inverted ethidium bromide stained agarose gel.

Likewise, in Figure 16 we compared the strains found among clinical samples with those found in fecal samples of healthy humans and found that the clinical samples are all similar among themselves while the fecal samples were extremely diverse.

Strain diversity of Enterococci isolates healthy humans and Clinical samples

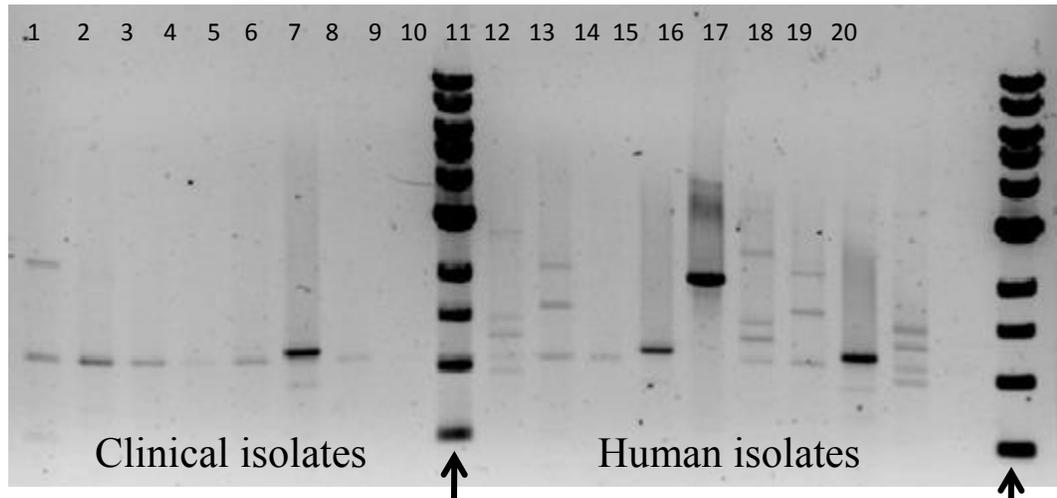


Figure 16. Box PCR describing the diversity and similarities of strains of Enterococci isolated from healthy humans and clinical samples using the primer BOXAR1. We show the representative dominant strains. Arrows denote 1Kb ladders. Inverted ethidium bromide stained agarose gel.

Interestingly, it appears that the clinical isolates are shared with some of the human fecal samples as seen in lanes 6 and 17, as well as in lanes 2-5 and 12.

Antibiotic resistance phenotypic and genotypic characterization: The antibiotic resistance pattern was determined using 166 isolates (15 from 12am; 27 from 4am; 21 from 8am; 12 from noon; 16 from 4pm; 31 from 8pm; and 44 from 12amII) obtained from the beach samples that were collected every four hours for 24 hrs. In Figure 17 we observe the results of our analysis. Most of our isolates regarding the time of sampling were resistant to Rifampicin while they were more susceptible to Piperacillin. Even though we observe a limit exceedence at 8pm and midnight we did not observe a difference in the antibiotic resistance pattern of the isolates assayed.

**Antibiotic Resistance Patterns during a 24 hour period
in enterococci isolates from a beach**

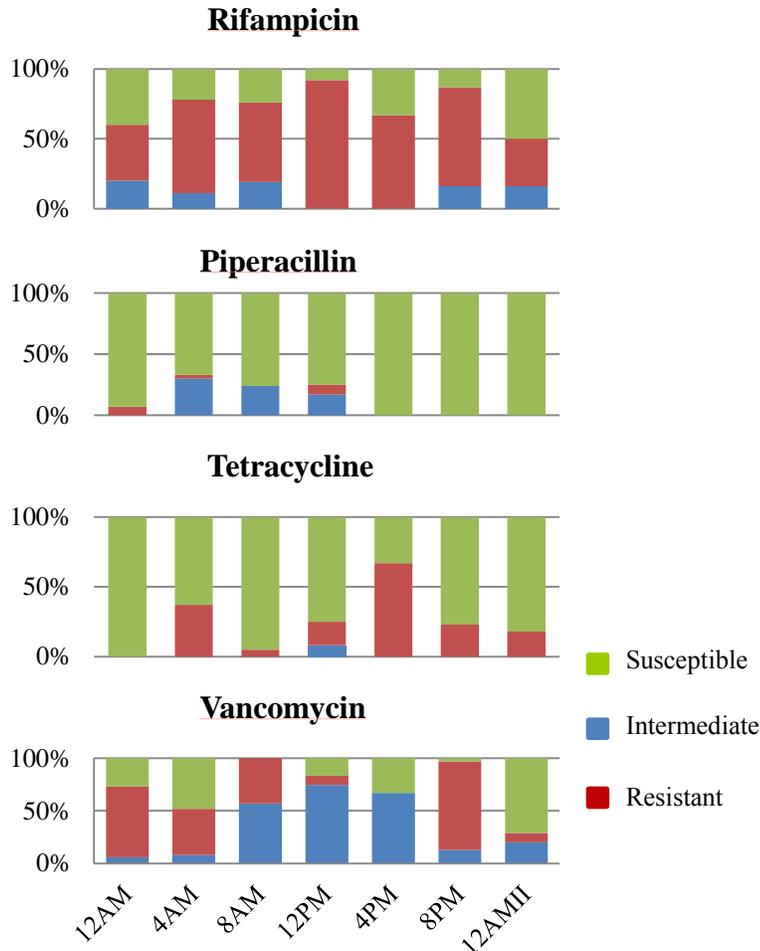
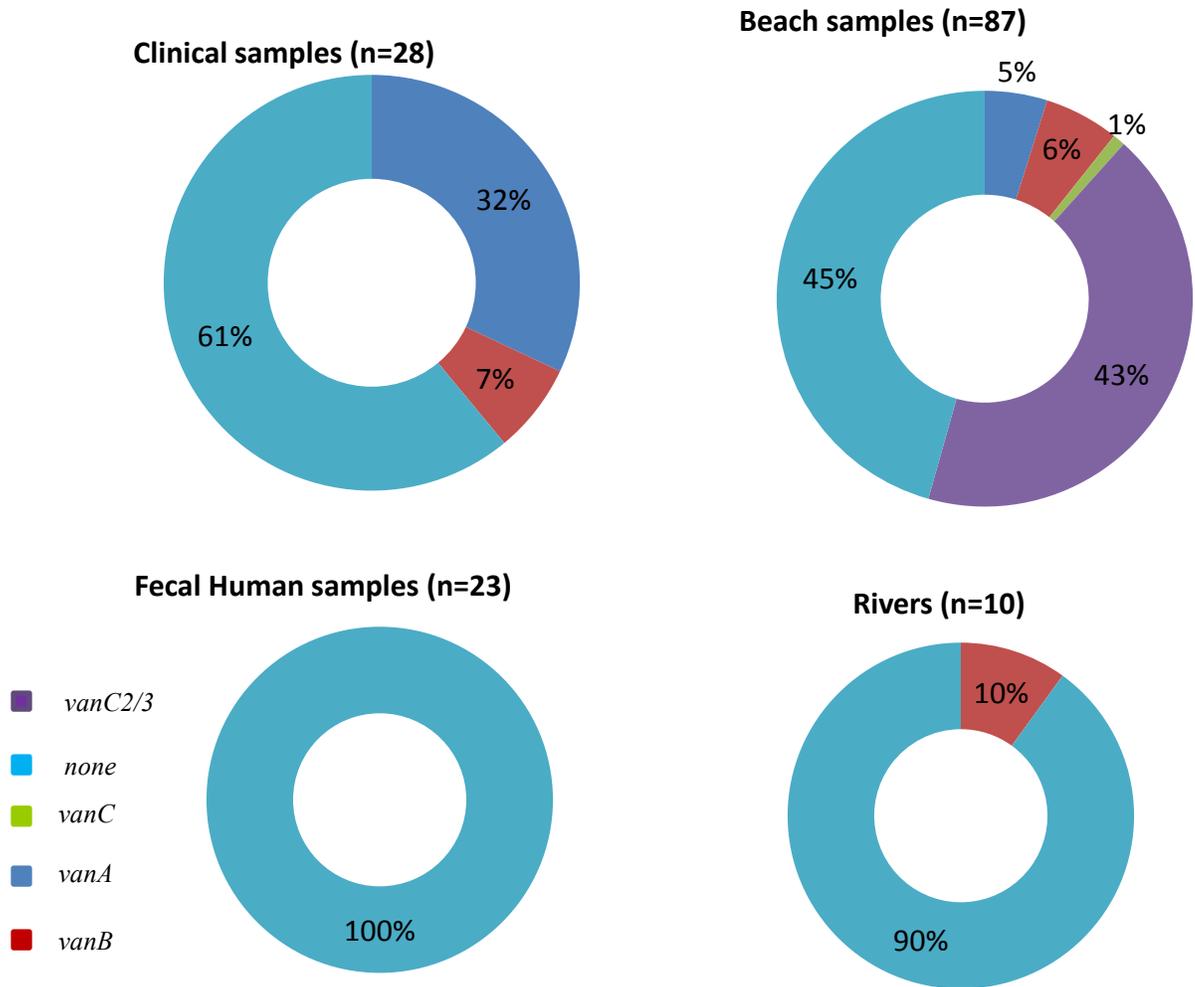


Figure 17. Antibiotic resistance determined by the Kirby-Bauer method using disk antibiotics: Rifampicin (5µg), Piperacillin (100µg), Tetracycline (30µg), and vancomycin (30µg).

Furthermore, based on our results and to understand the highest possible health risk to the public we decided to determine the genotype of the Enterococci isolates with a resistant and intermediate phenotype using vancomycin. Below we see the results of 148 isolates from different locations. Interestingly, the beach samples are the most diverse with presence of all resistance genes and *vanC2/3* being among the dominant ones. The clinical samples have the highest percentage of isolates with *vanA* than any other location. Surprisingly, we were unable to identify the dominant genotype that confers the resistance observed in the phenotype using the Kirby-Bauer method in all the samples.



A summary of our findings are presented in the following table that compares the results among all of our different locations. Interestingly, the animals and the beach share the presence of *vanC2/3* gene while *vanA* is shared between clinical, beach and pig samples.

Source (n)	<i>vanA</i>	<i>vanB</i>	<i>vanC</i>	<i>vanC2/3</i>	<i>none</i>
Pigs (25)	4%			16%	80%
Goats (25)				20%	80%
Clinical (28)	32%	7%			61%
Beach (87)	5%	6%	1%	43%	45%
Rivers (10)		10%			90%
Healthy Human (23)					100%

DISCUSSION

The Enterococci with the virulent genotype *gelE/asaI* is isolated frequently in recreational waters from around the world. The significance of this finding might indicate that this environment selects for the same genotype. This genotype appears to confer the ability to survive and compete within this system. The origin of these organisms is diverse since we could find them within healthy human feces, septic tanks, rivers, marine samples, and even clinical samples. It appears to be limited to humans and human activity but it is also found in chicken feces but not in pigs, horses, or goats that were living within the same farm. The fact that it is only found in chickens is surprising due to the differences in the digestive systems and the temperature differences among mammals and birds. On the other hand, it seems logical that these organisms would be present in human fecal matter but our preliminary data suggest that the Enterococci population is not very stable the human GI tract. As seen in Figures 3 and 5, the population of Enterococci changes with time, not only the species but also their virulent genotype suggesting that the colonization is not permanent; it is actually constantly changing thus making the Enterococci a transitory microbial component of the human feces. Furthermore, we could witness the coexistence of more than one enterococcal species with different virulent genes, some with more than two, but it seems that these organisms do not exchange this genetic information suggesting that it is not required to survive within the human body. In addition we observed that only *E. faecalis* seems to poses virulent genes within the fecal samples of healthy humans and they are incapable of transferring those genes to other species, namely *E. faecium*. It is important to point out that the most common virulent factor in clinical isolates of *E. faecium* is *esp* which is not observed in human fecal matter or in extremely low frequency from other environments like the beach.

Interestingly, since the Enterococci seems to constantly be changing within the fecal samples it becomes imperative to determine if they are acquired from the environment via food, water, or perhaps recreational activity. It is not clear for us right now if the same happens in the intestines of animals. The fact that we found the same genotype (*gelE/asaI*) in enterococcal samples from PR, Hawaii, southern California, and Australia suggests that this phenomenon is not limited to PR and perhaps these organisms occur naturally and might be independent to human contamination. To further explore the possibility that the natural environment is selecting for this genotype we evaluated the isolates by Box-PCR a fingerprinting technique. To our surprise we found that some strains are shared between all locations around the world and that they all share the same genetic material. This might suggest that in order to survive and persist in this environment these organisms must share the same genetic material. Furthermore, we identified certain organisms that appear to be endemic of a particular location since we could not find it at a different location. Moreover, using the same technique we evaluated if any of these strains was also shared among humans and animals. To our surprise we could not identify similar strains between the beach samples and the samples from human or animal feces suggesting that these organisms belong in the environment. On the other hand, we did found similar organisms shared between healthy humans and clinical samples as well as between pigs and healthy humans. Caution must be taken when interpreting this data since the number of animals was limited for instance we were able to sample only one horse, 5 goats, 3 pigs, 8 chickens and 8 humans. Another

interesting result was that the beach isolates were extremely diverse when compared to humans while the animals appear to be a bit more diverse than humans. It is important to understand that the fecal sample of animals was pooled together into a composite and not kept as individual samples. This might bias the interpretation of the diversity of the animals since it is possible that like in humans each animal only contain one or two different strains or species. Similarly it is important to emphasize that shared strains between clinical and human feces suggest that pathogenic strains exists among the human healthy population which might pose a public health risk by serving as carriers or reservoirs for microorganisms responsible for nosocomial infections in hospitals.

Another piece of evidence taken into consideration was the antibiotic resistant phenotypic characterization of some of the isolates. Even though this analysis was limited to beach samples exclusively we were able to observe that rifampicin resistance is most common among beach isolates. We believe that this observation represent a mutant lineage that is selected by the environment by rendering the isolate fitter to survive in the beach environment regardless of the presence of the antibiotic. This kind of mutation was described by Enne and colleagues (2004) using *E. faecium* in laboratory experiments. Interestingly the best antibiotic appears to be piperacillin followed by tetracycline and vancomycin. More importantly is the fact that the resistance pattern among the enterococcal community did not change during the exceedence event observed during the times of 8 pm and midnight. This fact suggests that the exceedence could be caused by the resuspension of organisms within the system and not from foreign organisms newly introduce into the system. Furthermore, when we concentrated on vancomycin resistance and looked at the distribution of van genes among the isolates we found that *vanA* genes are dominant among clinical samples but are also present among pigs and beach samples. It is not surprising to see this gen well represented among clinical isolates since it confer high resistance to vancomycin, but it is worry some to find 5% of the isolates from the beach that carry this gene. Likewise, *vanB* was also prevalent in clinical samples as expected but to see 6% of the isolates from the beach with this genotype raises our concern. Not surprising was the fact that the most abundant genotype among animals (pigs and goats) and beach samples was *vanC2/3* since this gene is innate among pigmented enterococci. Finally, the most interesting result to us was that there is an unknown genotype of vancomycin resistance present in all sources, especially among healthy humans. We still do not know if this variant represents a new un-described genotype or a new genetic variant that affect the priming sites used by the primer sets used in this project.

New development:

Using the same fingerprinting technique (Box-PCR) and in collaboration with Donna Ferguson, Dr. Marek Kirks, and Dr. Warish Ahmed we were able to obtain Enterococci isolates from Southern California, Hawaii, and Australia, respectively. We limit our analysis to the isolates that were identified as *E. faecalis* and shared a virulent genotype (*asa1/geIE*). To our surprise we found preliminary evidence that shows that those organisms are the same strains of Enterococci and are found and shared around the world, suggesting that the environment selects for the same genotype o that the same genotype allows the isolates to survive in these environments.

Similarities among strains of Enterococci isolated from beaches around

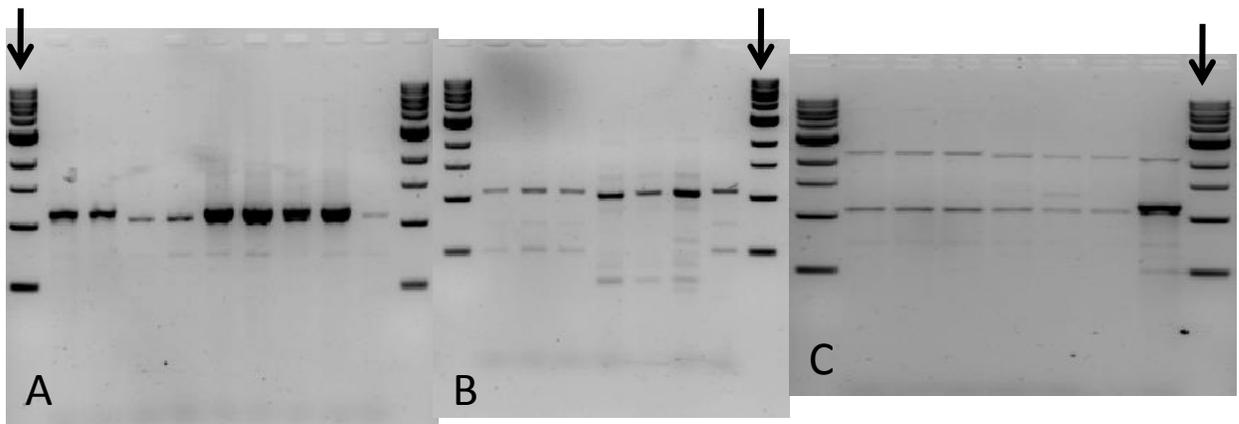


Figure 18. Box PCR describing the diversity and similarities of strains of Enterococci isolated from Puerto Rico, California, Hawaii, and Australia using the primer BOXAR1. Panel A, isolates from all countries. Panel B, isolates from Puerto Rico, Hawaii, and California. Panel C, isolates from Puerto Rico and Hawaii. We show the representative dominant strains. Arrows denote 1Kb ladders. Inverted ethidium bromide stained agarose gel.

Figure 19, summarizes the distribution of the different strains analyzed from our four locations around the world. It also shows endemic/unique strains in different locations. Furthermore it appears that the tropical islands share the most strains and that they are more diverse than the other two locations. The origin of these strains remains unknown!

Enterococcal strains with the same virulent genotype shared by geographical



Figure 19. Visual representation of the *E. faecalis* isolates with a genotype *asa1/gelE* and similar Box PCR from different geographical locations. The two tropical islands share the most strains. California, Australia,

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C) Describe opportunities for training, development and mentoring by project.

This research project has benefited our student's communication skills dramatically. Our students belong culturally to a Spanish speaking society even though our political background suggests that we are bilingual (Spanish/English). I emphasize in their scientific communication skills, time management, audiovisuals, interpretation of data, ability to answer questions, logical sequence of the presentation, etc.

The everyday activities in the laboratory allow me to teach them interpersonal skills, lab management skills, lab citizenship skills, problem solving skills, math skills, analytical skills, as well as work and science ethics. More importantly, I try to be a role model for all of my students by example. I try to let them know that I 'm not better than anybody in the lab or above any rules. I am simply more experienced! I try to create a relaxing, easy going, tolerating, patience, but strict scientific atmosphere.

Products of research efforts: Scientific communications:

A. U.S. Recreational Water Quality Criteria: A Vision for the Future - held March 11-13, 2013, Honolulu, Hawaii.

Poster Title: False alarms caused by EPA 1600 method in subtropical beach waters. **Luis A. Ríos Hernández***, Ginamary Negrón Talavera, José A. Romeu, Germán J. Rivera Castellar, Getzabeth E. Bosque Gómez and Michael I. Rivera Morales

B. Undergraduate Research Symposium, May 4, 2013. UPRM, Biology Department.

Poster Title: The marine environment selects for Rifampicin resistant Enterococci. **Mara Cuebas and Luis A. Rios Hernandez**

Poster Title: The effect of virulence factors and sugar availability on enterococcal biofilm formation. **Magdalis Gonzalez and Luis A. Rios Hernandez**

Poster Title: Distribution of Antibiotic Resistance Patterns and Vancomycin Resistance Genes in Enterococcus Spp. Isolated from Clinical, Human Fecal, Animal Fecal, River and Beach Samples. **Valery Lozada and Luis A. Rios Hernandez**

Poster Title: The Dominant Population of Enterococci in Fecal Samples of Healthy Humans is Equipped with the Same Virulence Factors Present in Disease-Causing Enterococcal Strains. **Getzabeth Bosque and Luis A. Rios Hernandez**

Poster Title: Population Diversity of Enterococcus Spp. in Poultry Fecal Samples. **Michael Rivera and Luis A. Rios Hernandez**

Poster Title: Comparison and Dominance of Enterococci Strains Sharing the Same Virulent Genotype from Different Aquatic Environmental Isolates Using a Finger-Printing PCR Technique. **German Rivera and Luis A. Rios Hernandez**

C. Congreso Internacional de Universidades Promotoras de la Salud y IV Conferencia Puertorriqueña de Salud Pública – Centro de Convenciones, San Juan, PR – 19 de marzo de 2013

Poster Title: Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. **Mara Cuebas and Luis A. Rios Hernandez**

D. Annual Biomedical Research Conference for Minority Students (ABRCMS)

Poster Title: The Antibiotic Resistance of Enterococcus spp. isolated from Natural Environments, Clinical, Healthy Humans, and Septic Tanks in Puerto Rico. **Mara Cuebas and Luis A. Rios Hernandez** 2012 at San José, CA.

E. American Society for Microbiology, May 18-21, 2013 at Denver, CO.

Poster Title: Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. ***Mara Cuebas and Luis A. Rios Hernandez***

Poster Title: Distribution of Antibiotic Resistance Patterns and Vancomycin Resistance Genes in Enterococcus spp. Isolated from Clinical, Fecal, River, and Beach Samples. ***Valery Lozada and Luis A. Rios Hernandez***

F. FEM-VI Frontiers in Environmental Microbiology - Mar 15, 2013 Universidad del Turabo, Gurabo, PR.

Poster Title: Using Phenotypic and Genotypic Differences among the Culturable Populations of Enterococci Through Event in Recreational Waters of Mayagüez, Puerto Rico Time to Predict a Recent Contamination. ***Mara Cuebas and Luis A. Rios Hernandez***

The development of human resources:

I feel confident that my students are well trained and could easily work at EQB, EPA, and Department of natural resources or any other agency that requires this expertise. I am sure that my students could (if they desire) pursue a graduate degree at any institution around the world. Their ability to think by themselves, analyze data, design experiments, keep records and present their work is at the level of any demanding academic institution or government agency. Notice that the majority of my students are minority females in STEMS, so I am contributing to educate and prepare females to succeed in science careers.

AN INTEGRATED APPROACH FOR THE DETECTION OF ESTROGENIC ACTIVITY IN A TROPICAL URBAN WATERSHED

Basic Information

Title:	AN INTEGRATED APPROACH FOR THE DETECTION OF ESTROGENIC ACTIVITY IN A TROPICAL URBAN WATERSHED
Project Number:	2012PR140B
Start Date:	3/1/2012
End Date:	2/28/2014
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Congressional District:	N/A
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Focus Category:	Surface Water, Water Quality, Toxic Substances
Descriptors:	
Principal Investigators:	Jorge R Ortiz-Zayas

Publication

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FINAL ANNUAL REPORT: Year 1

PROJECT: AN INTEGRATED APPROACH FOR THE DETECTION OF ESTROGENIC ACTIVITY IN A TROPICAL URBAN WATERSHED

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DATE: February 23, 2013

Modernity has arrived with important advances but has also introduced new environmental challenges, especially those related to the pollution of aquatic resources. Rivers are receiving increasing inputs of treated and untreated industrial and domestic wastewater with urban rivers more impacted. Urban streams have an important part to play in urban ecosystems because their position in the landscape makes these ecosystems particularly vulnerable to impacts associated with the urban environment. The Urban Stream Syndrome (USS) is a term used to describe a consistent pattern of hydrological, physical, and biological conditions seen in aquatic ecosystems downstream of urban inputs (Walsh et al., 2005). Research suggests that the two primary causes of the USS are storm water runoff and wastewater treatment plant effluents (Grimm et al., 2008; Paul and Meyer, 2001; Walsh et al., 2005). Compared to pristine streams, urban-impacted streams tend to receive larger loading rates of man-made organic chemicals such as PCB's and pharmaceuticals (Kolpin et al., 2002). Increased concentrations and loads of several chemical pollutants appear universal in

urban streams, often occurring even at low levels of catchment urbanization (Hatt, Fletcher, Walsh and Taylor, 2004).

Currently, more than 80,000 chemicals are in use and 2,000 are introduced each year in the United States (NIEH, 2010) with little or no data on its toxicological effects. Data from the most recent report of the Toxics Release Inventory (TRI) Program under the US Environmental Protection Agency (US EPA), revealed that 4.8 billion pounds of chemicals were released to the environment in the United States and its territories during 2009, including Puerto Rico, who showed a contribution of approximately 5.3 million pounds of the total amount registered nationally (US EPA, 2011). The US EPA in PR has included approximately 217 sites in the Comprehensive Environmental Response Compensation and Liability Information System (CERCLIS), and 19 of these are included in the National Priority List (NPL) for immediate cleaning and monitoring based on risk assessment studies (US EPA, 2011). The main contaminants found in the NPL for PR include heavy metals, volatile organic compounds, and pesticides known to cause effects to different organ systems in both humans and aquatic wildlife (US EPA, 2011). Among the reported contaminants there are known endocrine disruptors showing steroid-like properties such as bisphenol-A (Sonnenschein and Soto, 1998; Wagner and Oehlmann, 2010) and trichloroethylene (Wu and Berger 2007; Xu et al., 2004; Goh et al., 1998). The endocrine and reproductive effects of these chemicals are believed to be due to their ability to: (1) mimic the effect of endogenous hormones, (2) antagonize the effect of endogenous hormones, (3) disrupt the synthesis and metabolism of endogenous hormones, and (4) disrupt the synthesis and metabolism of hormone receptors (Sonnenschein and Soto, 1998).

Concern exists that many environmental and industrial chemicals may interfere with the endocrine system of both humans and wildlife thus naming them endocrine disrupting compounds (EDCs) (Colborn and Thayer, 2000). EDCs have been found to be present in surface waters and often at mixtures of high concentrations of low-potency disruptors and low amounts of very powerful ones (Focazio et al., 2008), making both wildlife and humans are at risk of exposure. **They are capable of interacting with the estrogen receptor in the cell causing effects even at trace-level concentrations** (Diamanti-Kandarakis et al., 2009; Kortenkamp, 2008). Effects include alteration of the normal biological signaling that control development (Colborn et al., 1994), and

reproduction (Anway et al., 2005; Barber et al., 2007; Cooper and Kavlock, 1997), among other internal functions controlled by the endocrine system.

EDCs are found in many of the products that people use everyday such as pharmaceutical drugs, plastics, cosmetics, detergents, and pesticides. EDCs often emerge in municipal wastewater supplies. For example, many pharmaceutical drugs excreted by people end up in the municipal wastewater system. Detergents used for washing clothes also drain into the same systems. In addition, EDC's can be transported through runoff from lawns, farmland or feedlots and industrial wastewater. The failure of wastewater treatment plants (WTP) to filter out EDC's leave them free to interact with fish, humans and other organisms that may ingest them downstream.

In 2002, the USGS published results from its first national reconnaissance of pharmaceuticals, hormones, and other organic wastewater contaminants in streams from the contiguous US. Results show that a wide range of chemicals are present in most streams, and that substantial levels of hormones, detergent metabolites (APEOs), plasticizers such as phthalates, and nonprescription drugs are common (Focazio et al., 2008; Kolpin et al., 2002). EDCs enter aquatic ecosystems mainly through water treatment plant (WTP) effluents. A large contribution is made by discharge of wastewater effluent from sewage treatment facilities, particularly for chemicals such as pharmaceuticals drugs for humans (Harries et al., 1996; Ternes et al., 1999). Natural and synthetic hormones and certain industrial chemicals capable of estrogenic effects have been identified in sewage effluents (Desbrow et al., 1998; Solé et al., 2000). Another source of EDCs to aquatic environments is the runoff from impervious surfaces in urbanized streams. Urbanization and changes in land use can have profound impacts on runoff characteristics bringing elevated concentrations of contaminants (Meyer et al., 2005). Conversion of land use from rural to urban can affect stream ecosystems via multiple pathways, including altered hydrology, water chemistry, channel geomorphology, and trophic resources (Paul and Meyer, 2001). Hydrological alterations include increased total runoff, shorter duration and higher volume peak runoff, and changes in base flows (Chadwick et al., 2006). Water quality is degraded in urbanized streams by both point and non-point sources of contaminants (Grimm et al., 2008; Kolpin et al., 2002; Paul and Meyer, 2001). Clearly, effects of catchment urbanization on stream ecosystems arise from many interrelated sources but catchment

impervious cover, however, has been shown to be a powerful proxy indicator that accounts for many of the factors mentioned earlier (Chadwick et al., 2006).

Areas with high population density obviously tends to imply more pollution, due to greater volumes of material and energy throughput arising from human activities (Eriksson and Zehaie, 2005). Generation and transport of pollutants are well studied because of requirements for compliance with national quality standards (see www.epa.gov/safewater/.html). However, we do not know how the effects of water pollution with EDCs from different human activities vary according to a spatial context. The connectivity framework provides a useful context for understanding how pollution-generating activities affect nearby water bodies and how much risk this pose to humans and wildlife.

The project "An Integrated Approach for the Detection of Estrogenic Activity in a Tropical Urban Watershed" aims to assess the presence of estrogenic activity in freshwater environments in Puerto Rico affected by human activities. The project was recommended for funding as part of the FY12 state competition sponsored by the Puerto Rico Water Resources and Environmental Research Institute (PRWRERI). On April 18, 2012, we received the official notification from the PRWRERI that the project was funded. A contract was signed by the UPR-Mayaguez and UPR-Rio Piedras authorities on May 16, 2012. The contract established the need to submit a biannual technical progress report and an annual final report. This report constitutes the annual final report submitted to the PRWRERI.

During the first year we proposed to focus on the Rio Piedras watershed by assessing potential estrogenic activity followed by identification of the potential compounds causing the activity. We have concluded the proposed four sampling events of the first year. Table1 lists the sampling dates and the physico-chemical parameters measured during the sampling events conducted at eight locations within the Rio Piedras watershed (Figure 1).

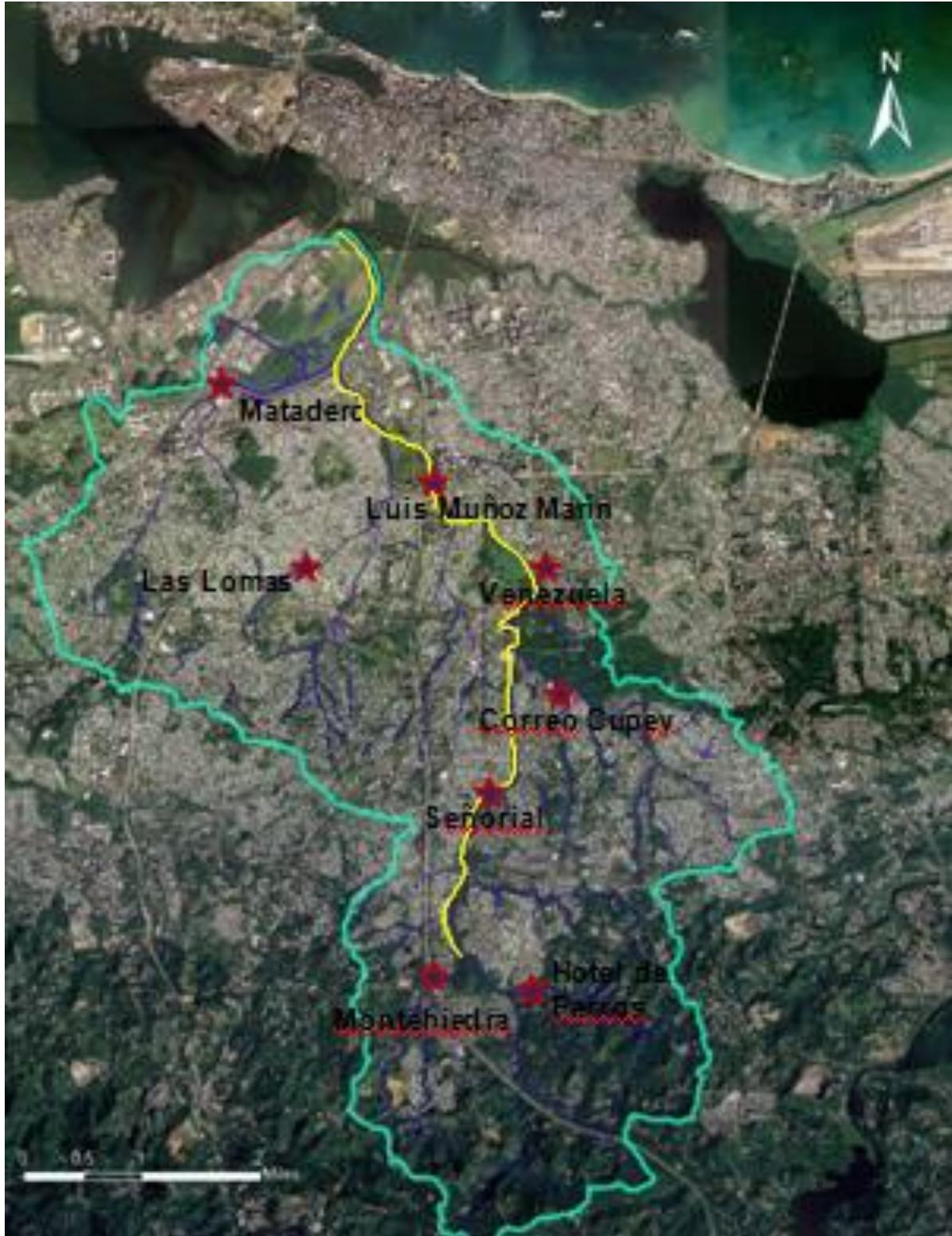


Figure 1. Map of the Rio Piedras Watershed, including the eight sampling stations of the San Juan ULTRA Project. Map obtained from sanjuanultra.org

These sites are part of the long-term monitoring program of the San Juan ULTRA Project, a pilot NSF project aimed at understanding the function of tropical, human-dominated systems. While the San Juan ULTRA water samplings occur every Tuesday, we collect samples for estrogenic activity analysis and chemical characterizations four times a year. Water samples are collected following standard protocols.

Eight sampling points located at different topographic levels (highlands, medium and lowland) in the watershed have been selected (Figure 1). These points are associated to the San Juan ULTRA Project (Urban Long Term Research Area). The sampling locations were established based on demographic gradients determined from census blocks level data from the 2000 Census (Seguinot and Hernandez, 2010). The San Juan ULTRA Project established a network of eight sampling stations in the Rio Piedras Watershed initiating a long-term water quality sampling program (Figure 1). This program is focusing at nutrients ($N + P$), organic carbon, common ions and physico-chemical parameters (discharge, dissolved oxygen, temperature, pH, specific conductivity, and total dissolved solids). We have used the existing sampling framework to collect additional water samples and test them for estrogenic activity (Figure 3). From our preliminary results, as we expected, it is observed that estrogenic activity augments downstream to the more populated areas. Along the watershed, sampling point at Barriada Venezuela shows the highest level of estrogenicity, $8.26E-11$ M. In this station, several point and non point sources of contamination have been identified such as illegal wastewater discharges and gray water discharges from the houses nearby the station. However, no estrogenic response was detected for sampling site Correo Cupey, which is a highly populated site. This underestimation could be explained by dilution of the estrogenic load in the surface water from local rainfall. In addition, that point is located in the confluence with a tributary which can exert the effect of dilution if no estrogenic load was present in its waters. Further re-analysis of this sample plus characterization with the GC/MS could overrule or confirm these explanations.

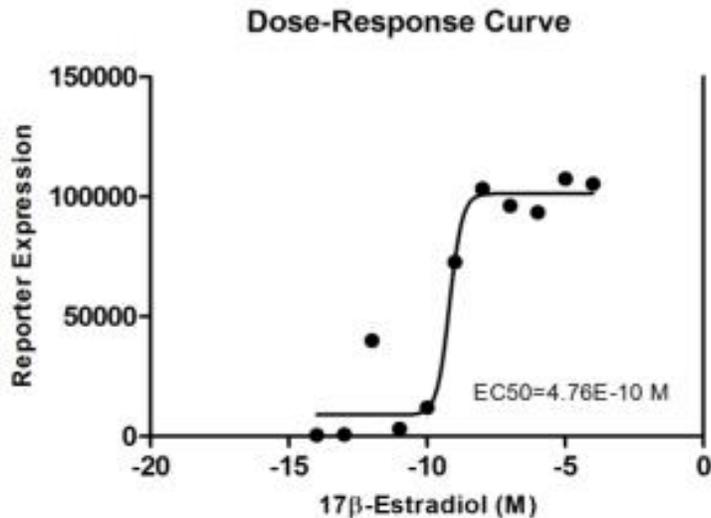


Figure 2. 17β-estradiol dose response curve for screening water samples. The resulting EC50 value averaged from three replicate experiments.

A *Saccharomyces cerevisiae* bioluminescent reporter assay was used to evaluate the estrogenic activity. The yeast strain has been engineered to have the human estrogen receptor (ERα) and it was kindly provided by Dr. Marc Cox, University of Texas at El Paso. The human estrogen receptor is activated when exposed to estrogenic compounds. Many non-steroidal substances have the ability to bind to the human estrogen receptor α (ERα) and to mimic the natural estrogen 17β-estradiol, many of these being xenoestrogens that are thought to disrupt the normal endocrine function in humans and animals (De Boever et al., 2001). A standard dose–response curve for 17β-estradiol (E2) was conducted based on the four-parameter logistic equation (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA, Figure 2). Water samples from the Rio Piedras Watershed show presence of estrogenic activity along the watershed with an increase in concentration as the gradient moves from a less to higher populated area (Figure 3, 4 and 5).

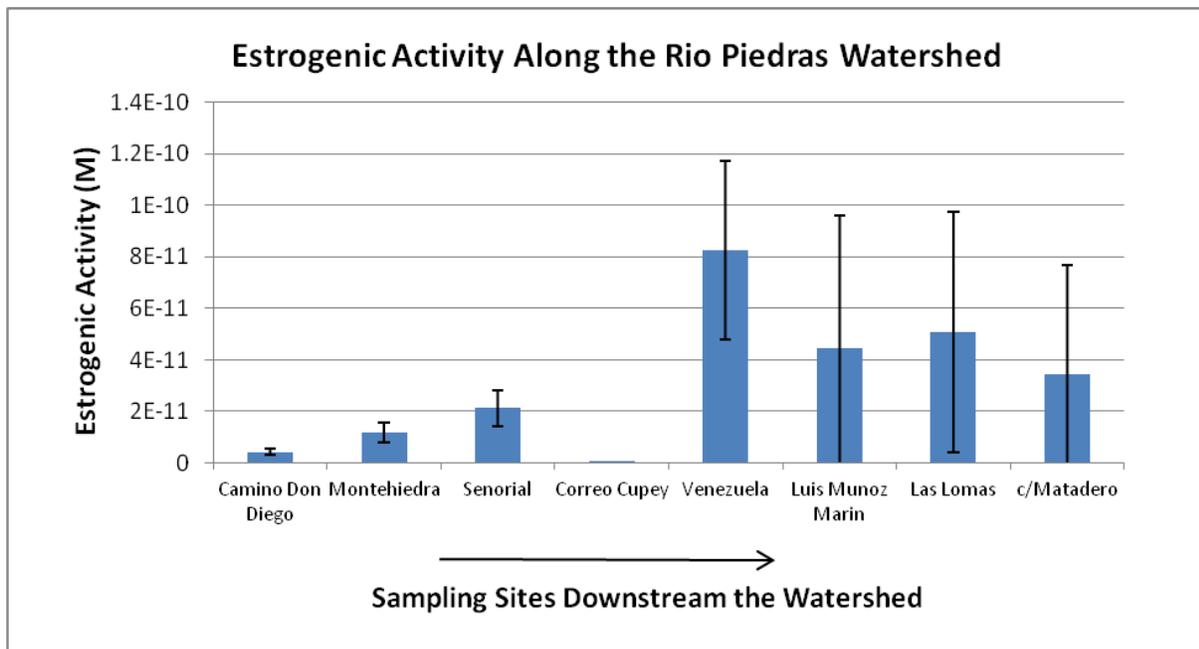


Figure 3. Preliminary results for estrogenic activity along the Rio Piedras Watershed from March-September, 2012. Shown are the mean values of estrogenic activity for each sampling site along the Rio Piedras Watershed. Sampling sites are located from left to right going from higher to lower elevation along the watershed.

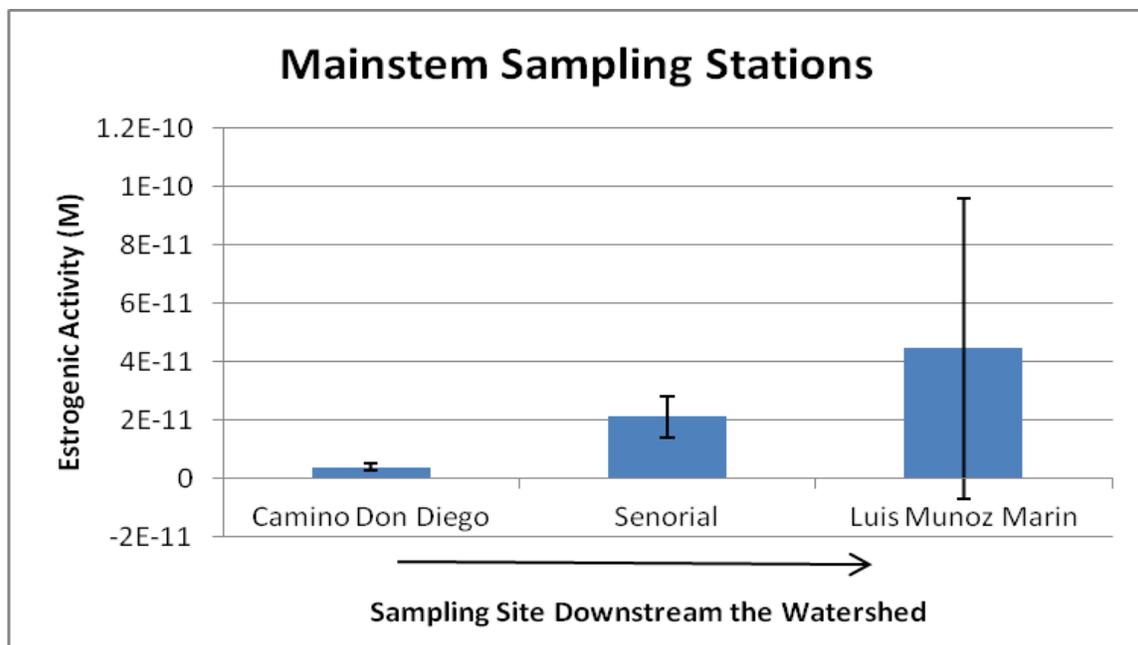


Figure 4. Shown are the mainstream sampling stations going downstream the watershed from the selected headwater Camino don Diego at Cupey to Luis Munoz Marin at Hato Rey. Estrogenic activity increases downstream the mainstream Rio Piedras.

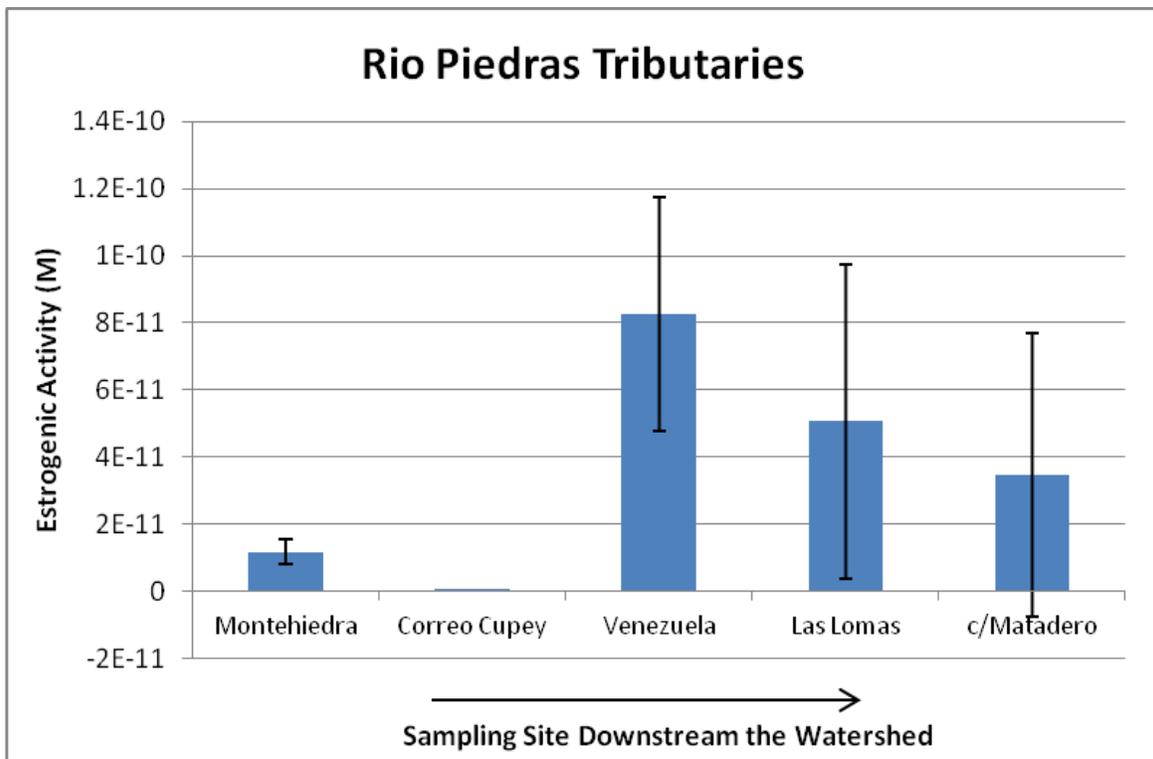


Figure 5. Rio Piedras tributaries showing estrogenic activity that contributes to the estrogenic load in the watershed.

Figure 6 shows how representative are our sampling dates in relation to the natural hydrologic dynamics of the Rio Piedras. The first sampling of March, 2012, recorded a flow of 0.15 m³/s, representing a per cent of exceedance of 99.8. This represents a dry season sampling period. The second sampling in June, 2012, recorded a flow of 0.91 m³/s with a % of exceedance of 42.6 and the third sampling occurred in September, 2012, and recorded a flow of 0.68 m³/s with a % of exceedance of 58.4. The fourth sampling occurred in January, 2013, for which no flow data is available due to vandalism to the USGS monitoring unit.

Table 1. Physico-chemical data collected during sampling events at the Rio Piedras.

Site	Date	Time	Temp (°C)	SpCond (uS/cm)	Salinity (ppt)	pH (s.u.)	TSS (g/L)	Discharge (m3/seg)*
Señ	3/20/2012	9:22	23.5	435.4	0.2	8.39	2.7	
HP	3/20/2012	10:01	23.5	315.2	0.2	7.76	11.4	
Mont	3/20/2012	10:35	24.4	440.5	0.2	8.03	14.5	
CC	3/20/2012	10:56	24.7	463.1	0.2	7.73	3.1	
Ven	3/20/2012	11:16	25.7	446.8	0.2	7.54	5.9	
LMM	3/20/2012	11:40	26.3	466.8	0.2	7.89	6.2	0.15
LL	3/20/2012	11:59	26.2	338.9	0.2	8.04	1.4	
Mat	3/20/2012	12:19	27.3	378.2	0.2	7.52	8.2	
Señ	6/19/2012	10:44	28.4	426.4	0.2	8.4	3.5	
HP	6/19/2012	11:05	26.7	268.8	0.1	7.88	14.2	
Mont	6/19/2012	11:26	27.5	425.5	0.2	8.16	2.9	
CC	6/19/2012	11:47	27.6	200.8	0.1	7.83	1.1	
Ven	6/19/2012	12:05	29.0	375.0	0.2	7.6	35.0	
LMM	6/19/2012	12:36	29.4	424.6	0.2	7.96	9.8	0.68
LL	6/19/2012	13:00	31.1	154.1	0.1	8.29	3.2	
Mat	6/19/2012	13:20	30.0	349.9	0.2	7.75	6.1	
Señ	9/18/2012	9:02	27.2	405.0	0.2	8.25	3.7	
HP	9/18/2012	9:21	26.6	325.8	0.2	7.57	3.8	
Mont	9/18/2012	9:45	26.9	417.2	0.2	7.92	4.0	
CC	9/18/2012	10:06	27.7	316.8	0.2	7.6	4.8	
Ven	9/18/2012	10:31	28.5	318.9	0.2	7.42	8.3	
LMM	9/18/2012	10:51	28.7	368.9	0.2	7.75	14.4	0.91
LL	9/18/2012	11:16	29.2	108.2	0.1	7.83	12.5	
Mat	9/18/2012	11:33	29.0	238.2	0.1	7.47	112.3	
Sen	1/15/2012	10:21	23.5	397.2	0.2	8.24	0.40	
HP	1/15/2013	10:38	23.4	287.4	0.1	8.81	34.9	
Mont	1/15/2013	11:02	23.8	311.3	0.1	8.15	2.20	
CC	1/15/2013	11:19	24.4	256.4	0.1	7.75	6.70	
Ven	1/15/2013	11:34	25.1	365.4	0.2	7.64	3.30	
LMM	1/15/2013	11:55	25.2	350.6	0.2	7.81	18.2	*
LL	1/15/2013	1:02	25.8	146.5	0.1	7.90	13.9	
Mat	1/15/2013	1:25	26.8	253.3	0.1	7.50	96.4	

Discharge data were collected by the USGS monitoring stations at Rio Piedras at Hato Rey (50049100).

*No data available

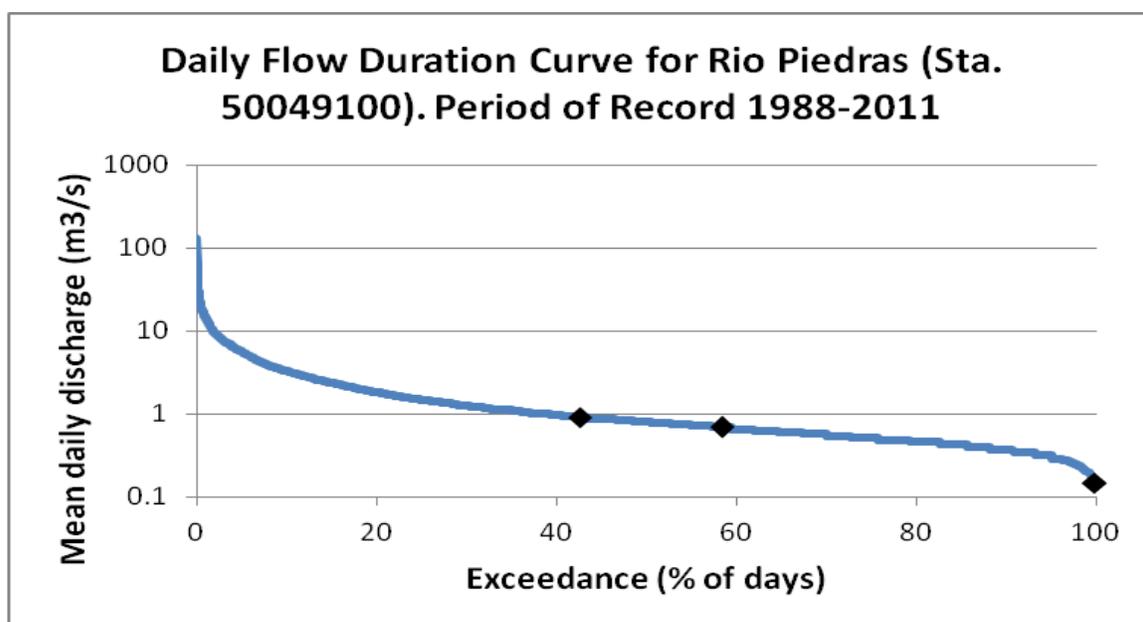


Figure 6. Flow duration curve for the Rio Piedras based on USGS data from 1988-2011. Highlighted are the flows of the river in the sampling dates. Flow data for the last sampling date is not available from the USGS.

This project represents, to our knowledge, the first attempt to study EDCs in Puerto Rico through bioassays of estrogenic activity. From our preliminary results, we are providing first evidence of estrogenic activity in the surface water of the Rio Piedras Watershed. Our preliminary findings are compatible with the hypothesis that surface water degradation occurs with estrogenic compounds as a population gradient increases in areas surrounding the water body.

Identification of potential estrogenic compounds in the collected water samples has been delayed due to a Gas Chromatography/Mass Spectrometry (GC/MS) malfunction. To overcome this, efforts have been made to establish collaboration with the UPRRP Materials Characterization Center (MCC). By the end of this February, 2013, training on the GC/MS has been finally arranged and it is our goal to start characterization of the samples by the end of March, 2013. Sampling of the Puerto Nuevo Primary Waste Water Treatment Plant (WWTP) and the Caguas tertiary WWTP has been completed. The water samples collected at the WWTPs and the Rio Piedras have been stored properly for GC/MS and estrogenic activity analyses.

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Information Transfer Program Introduction

Meetings, seminars, technical reports, 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 professional organizations in the region. All these activities facilitate the translation of research sponsored by the Institute into practical applications of direct benefit to industry, government, and the general public. Last year (FY-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 to organize the conference titled Water Resource Sustainability Issues on Tropical Islands. Other seminar and workshops have been offered as part of the various educational and technology transfer projects in collaboration with JBNERR.

USGS Summer Intern Program

None.

Student Support					
Category	Section 104 Base Grant	Section 104 NCGP Award	NIWR-USGS Internship	Supplemental Awards	Total
Undergraduate	9	0	0	0	9
Masters	3	0	0	0	3
Ph.D.	1	0	0	0	1
Post-Doc.	0	0	0	0	0
Total	13	0	0	0	13

Notable Awards and Achievements

Publications from Prior Years