Monitoring the diversity and species distribution of enterococci at a subtropical seawater system in Mayagüez, Puerto Rico

submitted by

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ABSTRACT

Enterococci are an important group of bacteria used as fecal indicator by the United States Environmental Protection Agency (USEPA) to evaluate the quality of recreational waters. Specifically, a beach is "contaminated" when the enterococci concentrations exceed the guideline of 104 CFU/100ml in a single sample analysis. Unfortunately, this method only provides numerical information and it lacks the differentiation of species and contamination sources. Given the establishment of this guideline in non-tropical regions and, due the frequent exceedance in tropical environments, we hypothesize that the USEPA enterococci guideline is not a reliable method to monitor the quality of recreational waters in Puerto Rico. In order to confirm this, first, we developed a new enterococci species identification method capable of discriminating adequately among environmental isolates using the RFLP technique of the *atpA* 1,102-bp fragment amplification. Then, this method was combined with a biochemical screening to confirm the isolates as *Enterococcus* and monitor the community in a beach water system every 4 hours for 24 hours. Even though our newly developed method has a limitation identifying *E. casseliflavus* isolates, we were able to identify non-pigmented enterococci species. It should be noted that three enterococci species were consistently identified: *E. faecalis, E. faecium* and *E. gallinarum.* Interestingly, our findings in the 24-hour study showed that the diversity of enterococci in the beach water system was principally limited to *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus* and other unknown species. These enterococci species were also identified during the "recent fecal contamination event" (before, during, and after), including the presence of five virulence factors (*gelE*, *asa1*, *hyl*, *esp*, and *cylA*) to compare the genetic makeup of the same enterococci species that were identified through the event. However, the presence of five virulence genes, limited to *E. faecalis* and *E. faecium*, had no significant variation when the enterococci

concentrations exceeded, or not, the USEPA standard. Our results showed that the enterococci community was extremely dynamic, acquiring and losing species diversity before, during, and after an exceedance event. If we consider our overall results, we might suggest that those exceedance events are not necessarily by a recent fecal contamination event. Finally, although further studies are needed to determine the source of enterococci introduction, fecal or not, we have to conclude that a single numerical datum is not a reliable method to identify the potential of health risks by fecal contamination in a tropical recreational water system.

RESUMEN

Los enterococos son un importante grupo de bacterias usados por la Agencia de Proteccion Ambiental de Estados Unidos (USEPA) como indicadores de contaminación fecal para evaluar la calidad de aguas recreacionales. Especifícamente, una playa está contaminada cuando las concentraciones de enterococos exceden el limite de 104UFC/100mL en un solo análisis. Desafortunadamente, este método sólo provee información númerica y carece de diferenciación de especies o fuentes de contaminación. Dado a que el establecimiento de este parámetro fue en regions no tropicales y, debido al frecuente incumplimiento en ambientes tropicales, hipotetizamos que el parámetro de enterocococos de USEPA no es un método confiable para monitorear la calidad de aguas recreacionales en Puerto Rico. Con la intención de confirmar esto, primero, desarrollamos un nuevo método de identificación de especies de enterococos capaz de discriminar adecuadamente entre los aislados ambientales usando la técnica de RFLP con el fragmento de amplificación de 1,102-bp del gen *atpA.* Luego, este método fue combinado con un filtro de pruebas bioquímicas para confirmar los aislados como *Enterococcus* y monitorear la comunidad en un sistema de agua de playa cada 4 horas por 24 horas. A pesar de que nuestro nuevo método desarrollado tiene limitaciones en identificar a *E. casseliflavus*, éste es capaz de identificar especies no pigmentadas. Cabe señalar que tres especies de enterococos fueron identificadas consistentemente: *E. faecalis, E. faecium* and *E. gallinarum.* Interesantemente, nuestros resultados en el estudio de 24 horas mostraron que la diversidad de enterococos en el sistema de agua de playa está principalmente limitada a *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus* y otras especies desconocidas. Estas especies de enterococos fueron también identificadas durante "el evento de contaminación fecal reciente" (antes, durante y después), incluyendo la presencia de cinco factores de virulencia (*gelE, asa1, hyl, esp,* and *cylA*) para comparar el marco genético de

las mismas especies que fueron identificadas a través del evento. Sin embargo, la presencia de los cinco genes de virulencia, limitados a *E. faecalis* y *E. faecium*, no tuvieron una variación significativa cuando las concentraciones de enterococos excedían, o no, el estándar de USEPA. Nuestros resultados mostraron que la comunidad de enterococos es extremadamente dinámica adquiriendo y perdiendo especies antes, durante y después de un evento de excedencia. Si consideramos nuestros resultados generales, podríamos sugerir que esos eventos de excedencia no son necesariamente debidos a un evento de contaminación fecal. Finalmente, aunque se necesitan estudios adicionales para determinar la fuente de introducción, fecal o no, tenemos que concluir que el uso de un dato númerico solamente no es un método confiable para identificar el potencial de riesgos a la salud por contaminación fecal en un sistema tropical de aguas recreacionales.

DEDICATION

To those hands that were available to help, regardless of the long days and nights…

To the friends, that shared a coffee and supported me unconditionally…

To my family, that always believed in me, more than I did…

To all those **"*You Can Do It!*"** that I received…

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Chapter One

Introduction, Study Goals and Objectives

1.1 Introduction

The enterococci are an important group of bacteria that have gathered a great interest by scientist due to their interaction with humans. The importance and concern for these organisms has increased especially due to two contradictory aspects: benefits and risks to the human community. Between these two points of view, some of the benefits included their role as gastrointestinal microflora in human and animals and as food fermenters. However, the most report of scientific contribution has been carried out to understand the risks that they impose for their increasing antibiotic resistance and the numerous virulence factors they possess (Jett et al., 1994; Mundy et al., 2000). In this context and considering the human exposure with enterococci by recreational activities on coastal water, quality assessments of the water should be conducted to ensure a safe enjoyment of the natural resources and to prevent health risks.

As an alternative to do it, the United States Environmental Protection Agency (USEPA) recommended the enterococci as fecal indicator bacteria (FIB) and they established guidelines to evaluate the quality of recreational waters (USEPA, 1986). Unfortunately, this method only provides numerical information but lacks information about species differentiation and possible fecal contamination sources. Therefore, the exceedance of the water quality guidelines represents an uncertain health risk to society, by assuming that all enterococci present in a sample are of human fecal origin. *Enterococcus faecium* and *E. faecalis* are the two enterococci that most frequently cause illness in humans, due to the pathogenicity of certain strains (McCormick et al., 2000). Although *E. faecalis* and *E. faecium* are the two enterococci most frequently associated with human disease, due the pathogenicity of certain strains, not all enterococci species are illness-causing.

At present, the USEPA enterococci guidelines of 104CFU/100ml are enforced by "Junta de Calidad Ambiental" (JCA; = Puerto Rico Environmental Quality Board, PREQB) in Puerto Rico, but typically they only report poor water quality by displaying a flag at the public beach, in their website, and a notification in the local newspaper. However, given the local enforcement establishment of these guidelines from non-tropical regions they can be considered as an unreliable alert method, especially by the frequent exceedance of the permissible levels in tropical environments (Shibata et al., 2004). Knowing this information, previous studies in the Hawaiian territories suggested that the USEPA guidelines are not appropriate for tropical islands (Fujioka et al., 1998). Recent works have suggested two possible reasons for the consistent exceedance in tropical waters: the survival (Bordalo et al., 2002) and the re-growth ability of the existent enterococci species (Desmarais et al., 2002).

Determining the long term persistence re-growth of enterococci is difficult when the regulatory standard is solely interpreted from a numerical level. However, studies by Anderson et al. (1997) suggested the persistence and replication of enterococci in the environment due to their high densities in marine sediments. Ferguson et al. (2005) and Yamahara et al. (2007), through studies conducted in Southern California, demonstrated that enterococci re-suspension and tidal action are responsible, in part, for the increasing concentrations of enterococci and the frequent beach water quality failures. Despite this information, there is still uncertainty regarding the detected enterococci with a possible source of contamination, whether natural or anthropogenic. However, the USEPA guidelines assume that the measurement of enterococci at elevated levels in seawater samples represent a direct fecal contamination event, but in reality it lacks the discriminatory power to differentiate between human and non-human sources of contamination.

1.2 Study goals

This study characterized the dynamics, dominance, and species distribution of enterococci within a subtropical beach environment. Our challenge was principally to develop an effective method of species identification. Finally, we hoped to assess the reliability of the USEPA enterococci standards in a subtropical environment in associating counts above the permissible levels with the frequency and abundance of species that potentially could cause illness in our recreational waters.

We hypothesized that the single use of the USEPA enterococci guideline is not a reliable method to monitor the quality of recreational waters in Puerto Rico.

1.3 Objectives

The main objective of this study was to identify the dynamics and distribution of enterococci species and to evaluate their reliability as fecal contamination indicators in seawater from subtropical environments.

The specific objectives for our research were:

1. Develop an effective procedure to identify the environmental enterococci to the species level.
2. Enumerate and monitor the community of enterococci in a seawater system during a 24 hours period.
3. Detect the presence of virulence factors among the community of environmental enterococci.

Chapter Two

Development of an effective procedure to identify the environmental enterococci to the species level

2.1 Introduction

The presence of enterococci in recreational waters indicates the presence of a pollution source that may contain human pathogens. It is important to recognize the source of contamination as a significant determining factor to evaluate the degree of health risks (Wiggins, 1996). For humans, health risks increase when people were exposed to human waste in comparison to animal waste (Sinton et al., 1993). In this context, the human-related species *E. faecalis* and *E. faecium* are considered the enterococci with greater potential to affect humans (Ruoff et al., 1990). Unfortunately, the USEPA 1600 method does not consider the identification of species profiles within the assessment of recreational water quality mainly due to time constraints, costs, and labor requirements.

Traditionally, biochemical tests have been the conventional alternative for the identification of enterococci by their negative reaction to catalase test, ability to hydrolyze esculin and to grow in 6.5% NaCl and at 45°C (Devriese et al. 1992). However, this biochemical characterization describes the genus *Enterococcus* and does not differentiate the more than 34 distinct species which vary on their pathogenic potential and source (Bergey & Boone, 2009). Given this limitation, Manero and Blanch (1999) proposed a biochemical key for the identification of enterococcal species. Unfortunately, this method is labor intensive and prone to errors due to the visual interpretation of the assays. Therefore, the development of genotypic techniques has been suggested as a more accurate option and cost effective than phenotypic methods (Moore et al., 2006).

The identification of the enterococci community using molecular techniques could be divided into taxonomic levels depending on the objectives of the researchers. For instance, for the

detection of the genus *Enterococcus*, Ke et al. (1999) proposed the Polymerase Chain Reaction (PCR) technique using the 112-bp fragment amplification of the elongation factor EF-Tu (*tuf* gene). This PCR based method is excellent to identify enterococci, but it is limited by the specificity of the primer set, which could amplify other organisms, thus requiring previous isolation and confirmation by biochemical tests. Given this limitation, Jackson et al. (2004) developed a multiplex PCR technique targeted at the 16S rRNA gene that facilitated identification to the genus level while also simultaneously establishing size differences of *sodA* (superoxide dismutase) genes allowing the identification of 28 species of enterococci. Unfortunately, this procedure is time consuming as it requires the set-up of seven different PCR reactions per DNA template sample.

As an easier alternative, comparative analysis of the 16S rRNA gene demonstrated phylogenetic evidence and the possibility to discriminate between enterococci species (Williams et al., 1991). However, the 16S rRNA gene amplification and sequencing revealed low discrimination potential among closely related species within groups like *E. faecalis* and *E. faecium* (Patel et al., 1998). To resolve this dilemma, the alpha subunit of the ATP synthase (*atpA*) gene sequence has been used as a reliable alternative with greater resolution power to discriminate between species (Naser et al., 2005).

In this chapter, we present a newly developed method capable of discriminating among environmental isolates. This method consist of the Restriction Fragment Length Polymorphism (RFLP) of the *atpA* gene that results in distinctive patterns that allows to identify the most abundant and dominant environmental enterococci in waterways of Puerto Rico. Furthermore, this method could be faster, cheaper, and easier than sequencing procedures, especially within educational and governmental laboratories with budget constraints.

2.2 Materials and methods

2.2.1. Microorganisms

We studied 938 enterococci isolates; among those, 842 were collected from our environmental studies, 57 were supplied by an anonymous local hospital, and 39 provided by Dr. Donna Ferguson (Southern California Coastal Water Research Project and University of Los Angeles, California). In addition, the following type culture were used as references: *Enterococcus faecium* ATCC 19434, *Enterococcus faecalis* ATCC 19433, *Enterococcus durans* ATCC 19432, *Enterococcus avium* ATCC 14025, *Enterococcus gallinarum* ATCC 49608, *Enterococcus casseliflavus* ATCC 51328, *Enterococcus sulfureus* DMS-6905, *Enterococcus aquimarinus* DMS-17690, *Enterococcus pallens* DMS-15690, *Enterococcus gilvus* DMS-15689 and *Enterococcus mundtii* NRRL B-51316 (USDA). Clinical isolates from a local hospital were isolated using Tryptic Soy Agar with 5% sheep's blood. Then, the presumptive enterococcal colonies were gram-stained and identified by a MicroScan system (Siemens Health Care). The environmental samples were collected and processed using the USEPA 1600 method, with the selective mEnterococcus medium (mE). Once enumerated, at least 10% of the total colonies the highest to 100% (per triplicate), were randomly chosen from nitrocellulose filters with sterile wooden toothpicks and transferred to Brain Heart Infusion Agar. Then, the genus confirmation (*Enterococcus*) was performed by biochemical tests and molecular techniques.

2.2.2. Biochemical tests

All presumptive enterococci isolates were first confirmed by biochemical tests that included: catalase reaction in 3% hydrogen peroxide, esculin hydrolysis in Bile Esculin Agar,

growth in Sulfate Indol Motility media (SIM), growth in Brain Heart Infusion Broth with 6.5% NaCl and at 45°C. Isolates with catalase negative reaction and a positive result in the rest of the mentioned tests were identified as *Enterococcus*.

2.2.3. Molecular techniques

In order to validate our genus identification, we first performed a DNA extraction with either of the methods that we compared: (i) bead-beating and (ii) total nucleic acid extraction with lysozyme. We amplified two genes (*tuf* and *atpA*) to confirm the affiliation with the genus *Enterococcus* based on biochemical tests. Finally, we used a double digestion of the *atpA* gene with the enzymes RsaI and AflIII (New England Biolabs) to develop our RFLP method to identify the enterococci species.

2.2.3.1. Bead-beating lysis

To recover the DNA in a faster and convenient way, we used the mechanically lysed by bead-beating cells from overnight cultures (12 hrs. of incubation) in Brain Heart Infusion Broth. Cells (1.5ml of the cultures) were centrifuged for 5 minutes at 13,000 rpm and the pellet was re-suspended in 200µl of 1X TE buffer, pH 8.0 (10mM Tris-HCl; 1mM EDTA). The samples were incubated at 95ºC for 10 minutes to destabilize the cell envelope. In order to extract the DNA, the samples were transferred to 2 ml screw cap tubes with 0.1g of 0.1mm glass beads previously sterilized and then vortex horizontally (Fisher Genie 2) for 5 minutes at maximum speed. Glass beads were removed by centrifugation for 5 minutes at 13,000rpm. The supernatant was transferred to a sterile microtube and stored at -20˚C.

2.2.3.2. Genomic DNA Extraction

As an alternate protocol of the bead-beating lysis, we extracted total nucleic acids from overnight grown cultures (12 hrs. of incubation) in Brain Heart Infusion Broth. First, we centrifuged 1.5ml of the cultures in eppendorf microtube for 5 minutes at 13,000 rpm (eppendorf 5415D), the supernatant was removed and the pellet was re-suspended in 500µl of cetyl-trimethyl ammonium bromide (CTAB) buffer [10ml 1M Tris (pH 8.4), 5ml 0.5M EDTA (pH 8.0), 28ml of 5M NaCl, 2g of CTAB, and 57ml ddH_2O]. Then, we homogenized and added 150µl of Lysozyme Solution (20mg Lysozyme/mL sterile ddH_20) and incubated the cells at 37°C /1 hour using a water bath. One volume of chloroform was added to the samples following a mixing with a full speed vortex (Fisher Genie 2) and centrifugation for 10min at 10,000rpm (Eppendorf 5415D). The supernatant was transferred to a new sterile 1.5mL Eppendorf microtube. Precipitation with one volume of 100% isopropanol was carried out overnight at -20ºC. After this, the DNA pellet was washed twice with 70% ethanol, air dryed, re-suspended in 100µl of TE 1:10 buffer (10 mM Tris–HCl, 0.1 mM EDTA; pH 8.1) and stored at -20°C.

2.2.3.3. *tuf* and *atpA* genes PCR

Ke et al. (1999) used a 112-bp fragment of the *tuf* gene to genus identification and Naser et al. (2005) the 1,102-bp fragment of *atpA* gene to identified enterococci as species levels. However, due to the unspecificity of these methods, prior confirmation of the enterococci by the biochemical tests is required. We evaluated both methodologies in our laboratory and decided to modify them to improve the results in the PCR amplification products. Basically, we developed the same PCR mixture for both methods, *atpA* gene (atpA-27-R, atpA-20-F) and *tuf* gene (Ent1,

Ent2), with their respective set of primers. The total of 50.0 µL PCR mixture was composed by 22.8µL Deionized Sterile Water, 10.0 µL (10X) Buffer (Promega), 5.0µL deoxynucleoside triphosphates (2.5mM each, New England Biolabs), 5.0 µL $MgCl_2$ (25mM, Promega), 2.5 µL forward and reverse primers (20µM each), 0.2µL Taq Polymerase (5U/µl Promega Flexi) and 2.0 µL of DNA template. For the amplification of *tuf* gene the thermal cycling conditions described by Ke et al. was used, however, cycling parameters for *atpA* gene (Naser et al., 2005) was modified as follows: 95°C for 3min; 35 cycles of 30 s at 95°C, 30 s at 49.6°C and 2min at 72°C; with a 10 min at 72°C final extension. Then, 10µL of the PCR mixture was resolved through 1.8% agarose-gel electrophoresis at 111v for 90 minutes. The gel was stained with ethidium bromide and the PCR product sizes were visualized by the molecular imager system VersaDoc MP 4000 (BioRad).

2.2.3.4. RFLP method

Once were confirmed by molecular and biochemical tests as enterococci, we generated a double digest method to distinguish and differentiate the enterococci isolates to the species level. To prepare the reaction, a 20µl of *atpA* PCR product was digested with 10 µl of the following digestion mix: 5.8µl Deionized Sterile Water, 3.0µl 10X NEBuffer 2 (New England Biolabs), 0.3µl BSA (100 µg/ml; New England Biolabs), 0.3µl RsaI (10,000 units/ml; New England Biolabs) and 0.6µl AflIII (10,000 units/ml; New England Biolabs). This reaction was incubated 2 hours at 37°C and then, at 80°C for 22 minutes to inactivate the enzymes. Finally, 25µL of the PCR digestion was resolved through 3.5% polyacrylamide-gel electrophoresis at 100v for 2.5 hours. The gel was stained with ethidium bromide and the digestion band patterns were visualized with the molecular imager system VersaDoc MP 4000 (BioRad).

2.2.3.5. Superoxide dismutase (*sodA*) gene Multiplex PCR

In order to validate the enterococci species identified by our RFLP method, we used the *sodA* gene Multiplex PCR (Jackson et al., 2004) with the following modifications to the master mix: 10.0 µL (10X) Buffer (Promega), 2.0µL deoxynucleoside triphosphates mix (0.2mM, New England Biolabs), 5.0 µL $MgCl_2$ (3mM, Promega), 5.0 µL 16mM (10x) $(NH_4)_2SO_4$, 1.25 µL forward and reverse primers (20µM each), 0.5µL Taq Polymerase (5U/µl Promega Flexi), 1.0 µL of DNA template and deionized sterile water to complete the 50µL final volume. Then, 20µL of the PCR mixture was resolved through 1.8% agarose-gel at 111v for 90 minutes. The gel was stained with ethidium bromide and the PCR product sizes were visualized by the molecular imager system VersaDoc MP 4000 (Bio-Rad).

2.3 Results

2.3.1. Genus-specific *tuf* gene PCR

After our biochemical screening, the 112-bp *tuf* amplification (Ke et al., 1999) was proposed to confirm the *Enterococcus* genus identification. However, beyond the expected results using enterococci positive controls (Figure 2.1: lanes 1-10) and environmental isolates (Figure 2.1: lanes 12-19), we observed 112-bp amplifications among non-enterococcal species. Specifically, as presented in the Figure 2.2, we used *Serratia marcescens* (lane 2), *Klebsiella pneumoneae* (lane 3), *Bacillus cereus* (lane 4) and *Staphylococcus aureus* (lane 5) to confirm the reliability of the method. However, we observed unexpected 112-bp amplifications with *Serratia marcescens* (lane 2) and *Staphylococcus aureus* (lane 5).

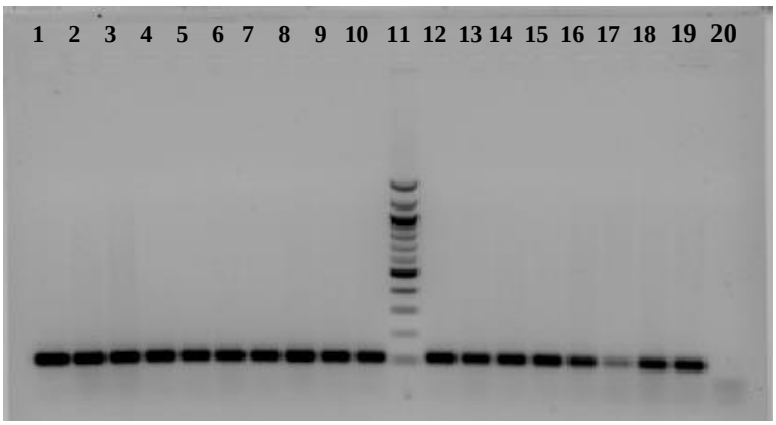


Figure 2.1. *Enterococcus* 112-bp *tuf* PCR. Lanes 1-10, positive controls; lane 1, *E. faecalis* ATCC 19433; lane 2, *E. faecium* ATCC 19434; lane 3, *E. durans* ATCC 19432; lane 4, *Enterococcus avium* ATCC 14025; lane 5, *Enterococcus gallinarum* ATCC 49608; lane 6, *E. casseliflavus* ATCC 51328; lane 7, *Enterococcus sulfureus* DMS-6905; lane 8, *Enterococcus aquimarinus* DMS-17690; lane 9, *Enterococcus pallens* DMS-15690; lane 10 *Enterococcus gilvus* DMS-15689; lane 11, 100bp molecular marker (Promega). Lanes 12-19, environmental isolates; lane 12, 12pm1; lane 13, 12pm2; lane 14, 4pm7; line 15, 4pm8; lane 16, 8pm3; lane 17, 8pm4; lane 18, 12amII 5; line 19, 12amII 6 and lane 20, negative control (no DNA template added).

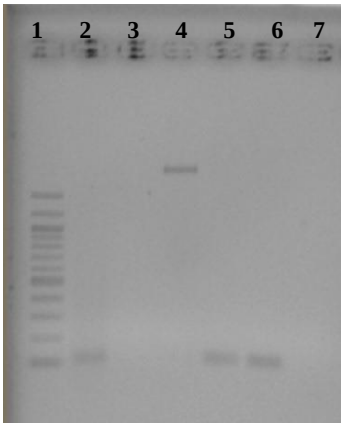


Figure 2.2. *Enterococcus* 112-bp *tuf* PCR. Lane 1, 100bp molecular marker (Promega); lane 2, *Serratia marcescens;* lane 3, *Klebsiella pneumoneae;* lane 4, *Bacillus cereus*; lane 5, *Staphylococcus aureus*; lane 6, *Enterococcus faecalis* and lane 7, negative control (no DNA template added).

2.3.2. Enterococci *atpA* gene PCR

According to Naser et al. (2005), using a 1,102-bp *atpA* gene fragment amplification can lead to identify *Enterococcus* spp. considering this, we tested his published PCR protocol as an alternative for species identification. As present in the Figure 2.3, we recovered the expected 1,102-bp band in most of our collection of enterococci controls (except *E. casseliflavus*: lane 4). However, some of them also presented unspecific bands: *E. faecalis* (lane 3) at 200-bp and 300b, *E. casseliflavus* (lane 4) at 290-bp, 490-bp and 601-bp, *E. gallinarum* (lane 6) at 200-bp and 450-bp and *E. avium* (lane 7) at 200-bp and 300-bp. Beyond being a problem, we preferred to interpret this issue as an advantage tool to distinguish between some enterococci species considering the reproducibility of this unspecific band in them. Testing this idea, we included some unknown enterococci isolates (lanes 2 and 9) also using some phenotypic characteristics to match them with a possible enterococci species. As a result, we predict that the non pigmented-non motile unknown (lane 2) could be *E. faecalis* (lane 3) or *E. avium* (lane 7). In contrast, the non pigmented-motile

unknown (lane 9) can be *E. gallinarum* (lane 6) whom also is the only enterococci species with these phenotypic characteristics.

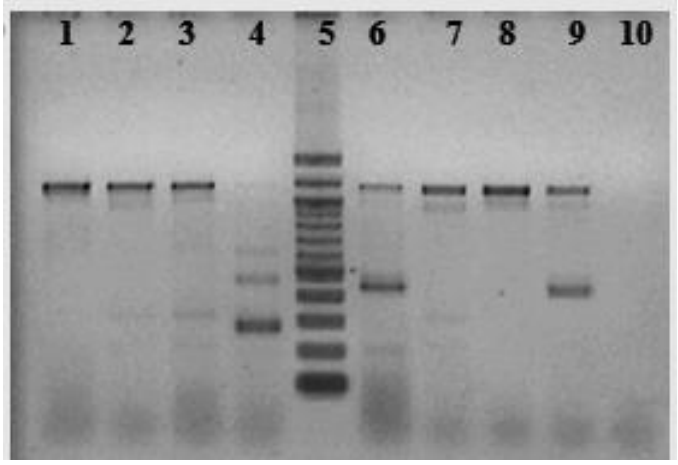


Figure 2.3. *Enterococcus* 1,102-bp *atpA* PCR. Lane 1, *E. faecium* ATCC 19434; lane 2, Unknown non pigmented-non motile enterococci; lane 3, *E. faecalis* ATCC 19433; lane 4, *E. casseliflavus* ATCC 51328; lane 5, 100bp molecular marker (Promega); lane 6, *Enterococcus gallinarum* ATCC 49608; lane 7, *Enterococcus avium* ATCC 14025; lane 8, *Enterococcus durans* ATCC 19432; lane 9; Unknown Non Pigmented/Motile enterococci and lane 10, negative control (no DNA template added).

2.3.3. RFLP method

Knowing that the *atpA* gene 1,102-bp fragment was not enough to identify enterococci species, we conducted RFLP (double digests) of *atpA* gene. In order to validate this method, it was tested using the same enterococci controls used in the previous *atpA* PCR (Figure 2.3). As shown in Figure 2.4, the double digestion generated fingerprints that allow differentiation of particular species and the identification of unknown strains (Figure 2.3, lanes 2 and 9) through these results. We could match perfectly the Non pigmented/Non motile unknown (lane 2) with *E. faecalis* (lane 3) and also, confirm the Non Pigmented/Motile unknown (lane 9) as *E. gallinarum* (lane 6).

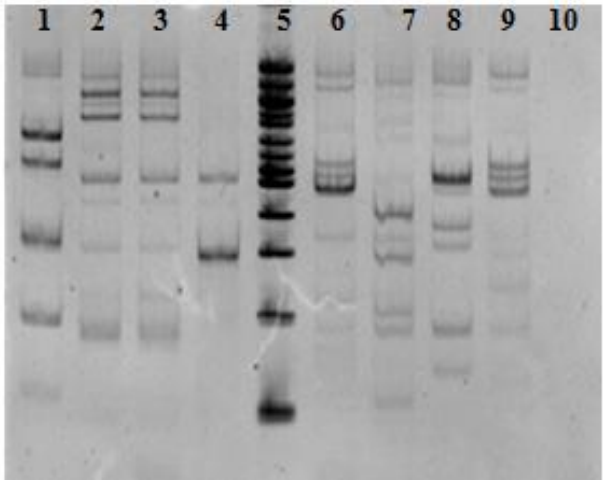


Figure 2.4. *Enterococcus atpA* gene RFLP. Lane 1, *E. faecium* ATCC 19434; lane 2, unknown non pigmented-non motile strain; lane 3, *E. faecalis* ATCC 19433; lane 4, *E. casseliflavus* ATCC 51328; lane 5, 100bp molecular marker (Promega); lane 6, *Enterococcus gallinarum* ATCC 49608; lane 7, *Enterococcus avium* ATCC 14025; lane 8, *Enterococcus durans* ATCC 19432; lane 9; unknown non pigmented-motile strain and lane 10, negative control (no DNA template added).

2.3.4. Limitations with the *atpA* PCR

Pigmented-motile environmental isolates, presumptively *E. casseliflavus*, failed to produce *atpA* amplicons of the expected size (1,102-bp) or yield non-specific PCR products (Figure 2.5; lanes 3-7). The same was observed for *E. casseliflavus* reference strain ATCC 51328 (lane 2).

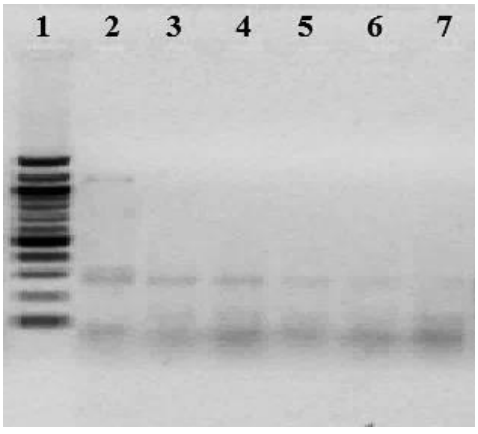


Figure 2.5. *Enterococcus atpA* gene PCR for pigmented-motile environmental isolates. Lane 1, 100bp molecular marker (Promega); lane 2, *E. casseliflavus* ATCC 51328, lane 3, 12am6; lane 4, 12am7; lane 5, 12am8; lane 6, 12am8; lane 7, 12am9.

2.3.4.1. Pigmented-motile isolates

Although *atpA* PCR limitations with pigmented/motile isolates were detected, we also tested these pigmented/motile isolates, presumptively *E. casseliflavus*, with the RFLP method. According to the results shown in Figure 2.6, no digestion occurred in the sample, as expected, because we did not amplify the *atpA* 1,102-bp in the PCR. Basically, what could be seen is the same unspecific 290-bp band that we observed in the *atpA* gene PCR (Figure 2.5).

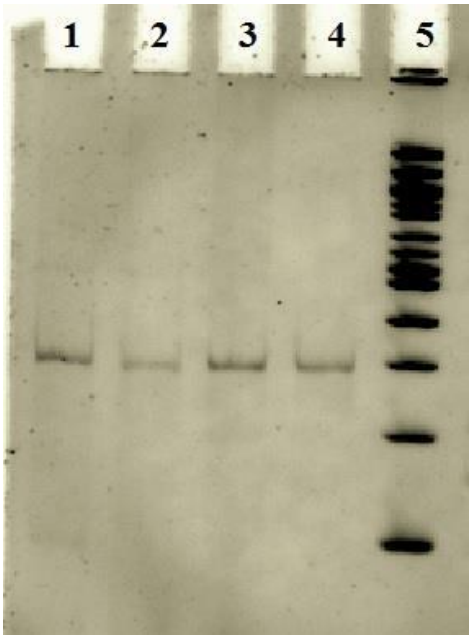


Figure 2.6. Pigmented/motile *Enterococcus atpA* gene RFLP. Line 1, *E. casseliflavus* ATCC 51328; line 2, 12am8; line 3, 12am6; line 4, 12am7; line 5, 100bp molecular marker (Promega)

According to this alternative, we assumed that this lack of specificity was due to genetic differences within pigmented strains, hence we studied several pigmented enterococci species other than *E. casseliflavus*. Some of them were: *E. sulfureus* DMS-6905, *E. aquimarinus* DMS-17690, *E. pallens* DMS-15690, *E. gilvus* DMS-15689 and *E. mundtii* (Donated by USDA). According to Figure 2.7, we found that the lack of the expected 1,102-bp *atpA* fragment occurred

in some pigmented enterococci species but not in all. Specifically, the *atpA* gene did not amplify for *E. sulfureus* (lane 3), *E. casseliflavus* (lane 4) and *E. gilvus* 1 (lane 6).

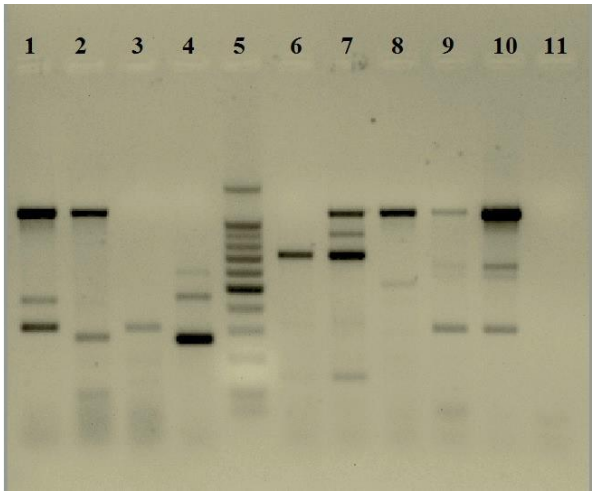


Figure 2.7. *Enterococcus* positive controls *atpA* gene PCR Line 1, *E. mundtii* (USDA); line 2, *E. pallens* DMS-15690; line 3, *E. sulfureus* DMS-6905; line 4, *E. casseliflavus* ATCC 51328; line 5, 100bp molecular marker (Promega); line 6, *E. gilvus* DMS-15689 #1; line 7, *E. gilvus* DMS-15689 #2; line 8, *E. aquimarinus* DMS-17690; line 9, *E. faecalis* ATCC 19433; line 10, *E. faecalis* MMH594; line 11, negative control (No DNA template added).

Analyzing the results shown in Figure 2.7, we also detected amplification differences between same enterococci species with DNA template extracted by different methods. Specifically, *E. faecalis* ATCC 19433 (lane 9) DNA template was extracted in our laboratory using a mechanical bead-beating method, in contrast with the *E. faecalis* MMH594 (lane 10) DNA template (kindly donated by N. Shankar, Department of Medicinal Chemistry and Pharmaceutics, University of Oklahoma Health Sciences Center, Oklahoma City) that was extracted using a genomic extraction with lysozyme (Shankar et al., 1999). Figure 2.8 presents the results we obtained testing each enterococci DNA template twice, using both methods. The use of bead-beating extraction (above, lanes 1-9) has the disadvantages of not yielding the expected 1,102-bp

atpA fragment (lanes 2, 3, 4, 5, 7, 8 and 9) or producing nonspecific amplifications (lanes 2, 3, 5, 7 and 9) in comparison with results from chemical lysis (below, 1'-9').

Figure 2.8. *Enterococcus* positive controls *atpA* gene PCR Bead-beating extraction (above, lanes 1-9). Lane 1, 100bp molecular marker (Promega); lanes 2 and 3, *E. casseliflavus* ATCC 51328; lanes 4 and 5, *E. sulfureus* DMS-6905; lanes 6 and 7, *E. pallens* DMS-15690; lanes 8 and 9, *E. mundtii* (USDA). Genomic extraction with lysozyme (below, lanes 1'-9'). Lane 1', 100bp molecular marker (Promega); lanes 2' and 3', *E. casseliflavus* ATCC 51328; lanes 4' and 5', *E. sulfureus* DMS-6905; lanes 6' and 7', *E. pallens* DMS-15690; lanes 8' and 9', *E. mundtii* (USDA).

DNA obtained by chemical lysis improved the amplification of the 1,102-bp amplicon relative to DNA from the bead-beating method to obtain better and brightly amplifications, we observed differences among similar species (lanes 2' and 3', 4' and 5', 6' and 7', 8' and 9') with this method. Specifically, was not consistently amplified and nonspecific products of a lower size were present in all instances. Possible explanations could be differences of the DNA template concentrations because we extracted separately each one of them. To corroborate this assumption, we tested several times the same *E. pallens* DMS-15690 DNA template extracted by chemical lysis and using dilutions to create different measured concentrations (Nanodrop, ND-1000). According with Figure 2.9, we demonstrate that the results for the PCR method using *atpA* gene are reproducible; however, the DNA template concentration could affect the results. Comparison of the highest concentration (lane 1) with lowest concentration (lane 10) demonstrated significant differences in clarity, brightness and amplification quality to distinguish unspecific bands.

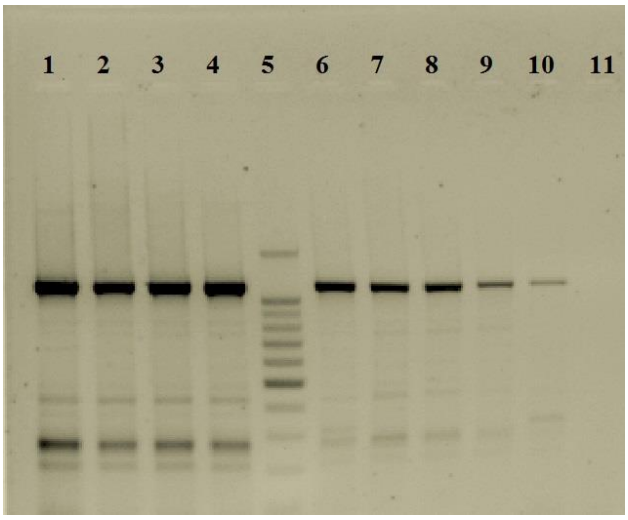


Figure 2.9. *Enterococcus pallens* DMS-15690 *atpA* gene PCR. Line 1, 1,030ng/µl; line 2, 500 ng/µl; line 3, 400 ng/µl; line 4, 200 ng/µl; line 5, 100bp molecular marker (Promega); line 6, 100 ng/µl; line 7,50 ng/µl; line 8, 25 ng/µl; line 9, 10 ng/µl; line 10, 5 ng/µl; line 11, negative control (No DNA template added).

Knowing the importance of the consistency to correctly identify the enterococci species through a band pattern, we investigated how these differences might affect our final results using RFLP. In Figure 2.10, our main interest was to compare RFLP results between a high measured DNA template concentration and a final, low, 10ng/µl diluted DNA template.

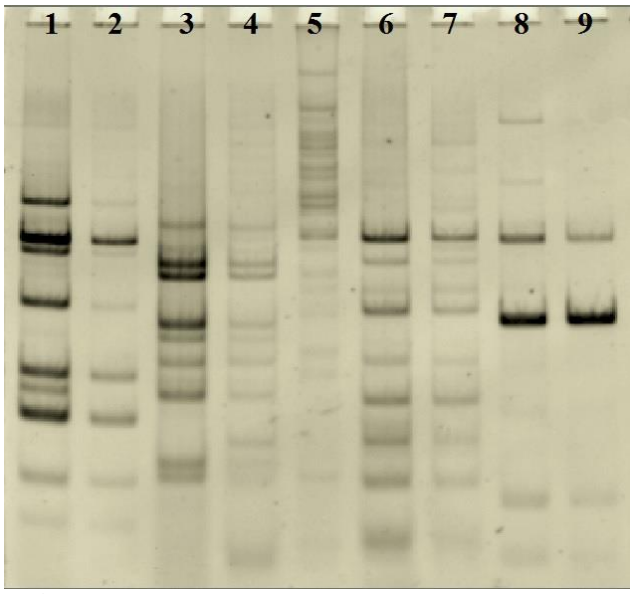


Figure 2.10. *Enterococcus atpA* gene RFLP. Lane 1, *E. pallens* DMS-15690 (1,030ng/µl); lane 2, *E. pallens* DMS-15690 (10ng/µl); lane 3, *E. mundtii* USDA (917ng/µl); lane 4, *E. mundtii* USDA (10ng/µl); lane 5, 100bp molecular marker (Promega); lane 6, *E. faecalis* ATCC 19433 (1,002ng/µl); lane 7, *E. faecalis* ATCC 19433 (10ng/µl); lane 8, *E. casseliflavus* ATCC 51328 (1,218ng/µl); lane 9, *E. casseliflavus* ATCC 51328 (10ng/µl).

According to the results, the main difference was the brightness, however, it was possible to recover the band patterns among the same species. Considering the problem of no digestion with *E. casseliflavus* (lanes 8 and 9), we decided to look at the reagents, specifically on the Taq DNA Polymerase. Promega Flexi Taq Polymerase was compared to New England Biolabs DNA Polymerase in order to detect differences in amplification due to the choice of polymerase (Figure 2.11) and the RFLP method (Figure 2.12).

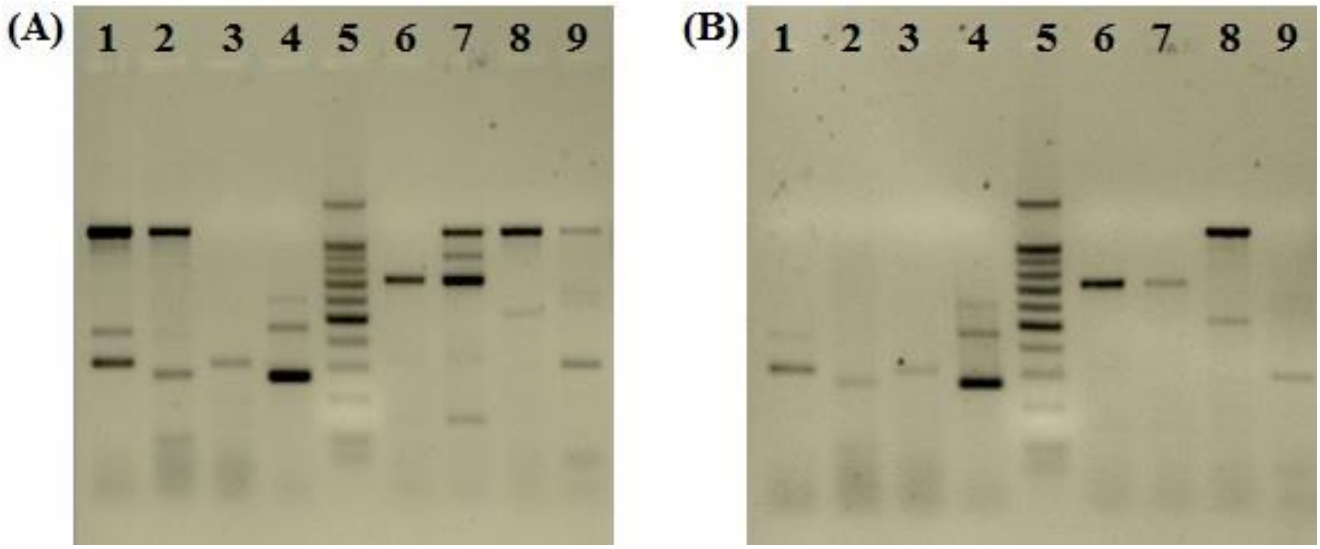


Figure 2.11. *Enterococcus atpA* gene PCR using Flexi Taq (A) and New England Biolabs (B) DNA polymerase. Line 1, *E. mundtii* (USDA); line 2, *E. pallens* DMS-15690; line 3, *E. sulfureus* DMS-6905; line 4, *E. casseliflavus* ATCC 51328; line 5, 100bp molecular marker (Promega); line 6, *E. gilvus* DMS-15689 #1; line 7, *E. gilvus* DMS-15689 #2; line 8, *E. aquimarinus* DMS-17690; line 9, *E. faecalis* ATCC 19433.

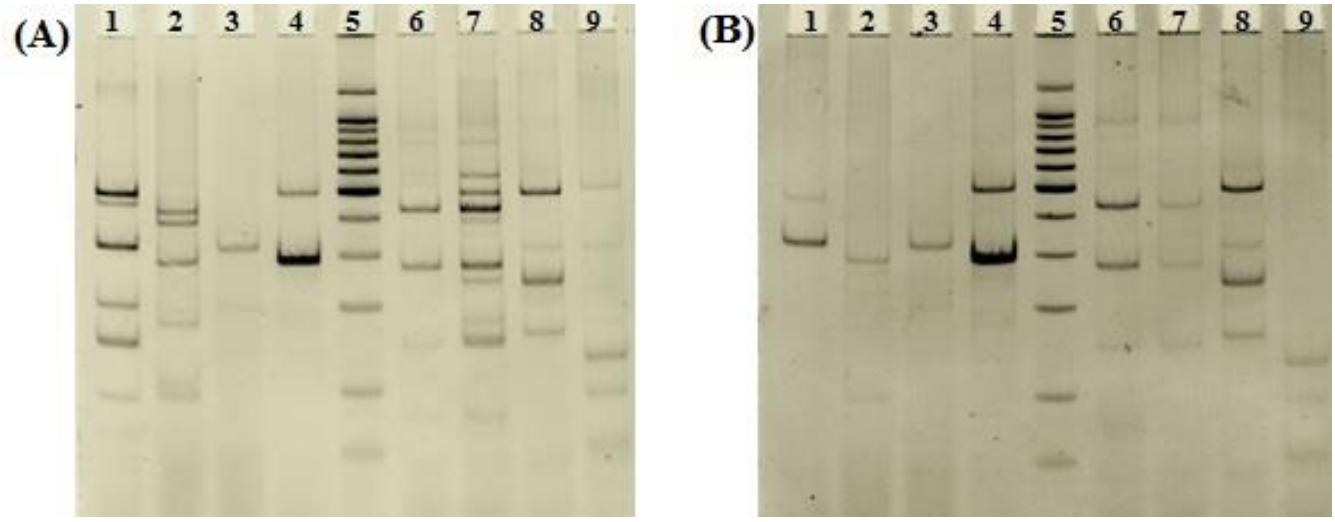


Figure 2.12. *Enterococcus atpA* gene RFLP using Flexi Taq (A) and New England Biolabs (B) DNA polymerase. Line 1, *E. mundtii* (USDA); line 2, *E. pallens* DMS-15690; line 3, *E. sulfureus* DMS-6905; line 4, *E. casseliflavus* ATCC 51328; line 5, 100bp molecular marker (Promega); line 6, *E. gilvus* DMS-15689 #1; line 7, *E. gilvus* DMS-15689 #2; line 8, *E. aquimarinus* DMS-17690; line 9, *E. faecalis* ATCC 19433.

The Promega polymerase (Figure 2.11-A) was more effective than New England Biolab Taq Polymerase. Since most amplification issues happened with the pigmented-motile strains, presumptively *E. casseliflavus*, we randomly picked some isolates of this phenotype. Specifically, we proved and noticed improved amplification with DNA obtained with chemical lysis and

amplified with Promega polymerase as most of the isolates, except lane 5 (Figure 2.13-A) shown the expected 1,102-bp *atpA* fragment. However, although all of them were supposedly *E. casseliflavus*, we observed different patterns when considering the unspecific bands. We found the following similarities through the *atpA* gene PCR results in some environmental isolates (Figure 2.13-A): **(a)** 8pm 22 (lane 1), 8pm 24 (lane 3), 8pm 37 (lane 9), 8pm 38 (lane 10), 8pm 39 (lane 11) and 8pm 40 (lane 12); **(b)** 8pm 28 (lane 7) and 8pm 29 (lane 29); **(c)** 8pm 23 (lane 2) and **(d)** 8pm 25. Similar groups were compared in the next step, the RFLP. According with Figure 2.13-B, lanes 1-5 represents group **a**, lane 7 group **c**, lane 8 group **d** and lanes 9-10 group **b**. Despite this, we observed that groups with more than one similar unspecific band **(a and b)** presented more than one band pattern results.

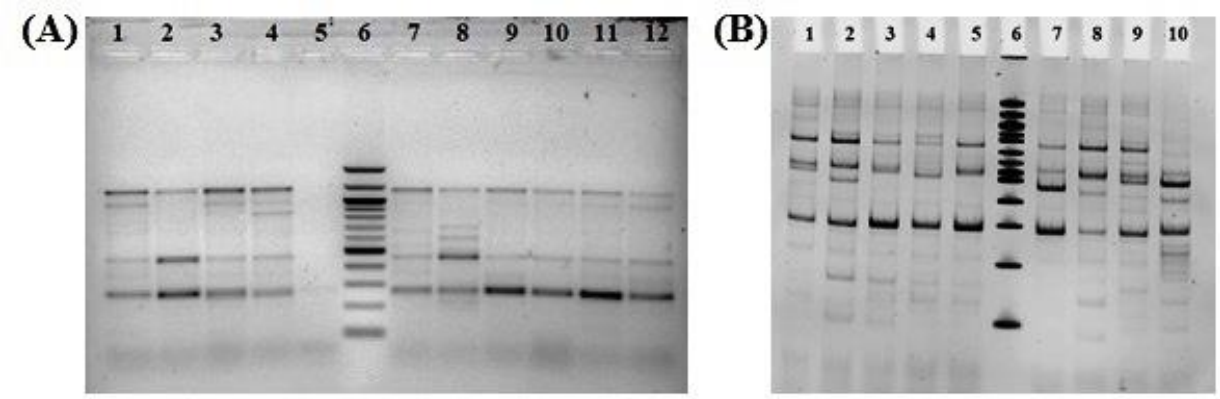


Figure 2.13. (A) *Enterococcus atpA* gene PCR and (B) RFLP with presumptively *E. casseliflavus*. (A) Lane 1, 8pm 22; lane 2, 8pm 23; lane 3, 8pm 24; lane 4, 8pm 25; lane 5, 8pm 27; lane 6, 100bp molecular marker (Promega); lane 7, 8pm 28; lane 8, 8pm 29; lane 9, 8pm 37; lane10, 8pm 38; lane 11, 8pm 39; lane 12, 8pm 40. (B) Lane 1, 8pm 22; lane 2, 8pm 24; lane 3, 8pm 39; lane 4, 8pm 40; lane 5, 8pm 37; lane 6, 100bp molecular marker (Promega); lane 7, 8pm 23; lane 8, 8pm 25; lane 9, 8pm 28; lane10, 8pm 29.

These results shown differences within the band patterns that clearly limit the identification process. However, we also confirmed that these pigmented-motile environmental isolates were *E. casseliflavus* using the 288-bp fragment of the *sodA* gene PCR as describe by Jackson et al., 2004. As shown in Figure 2.14, all the different band patterns presented in the Figure 2.13-B indeed belong to *E. casseliflavus*.

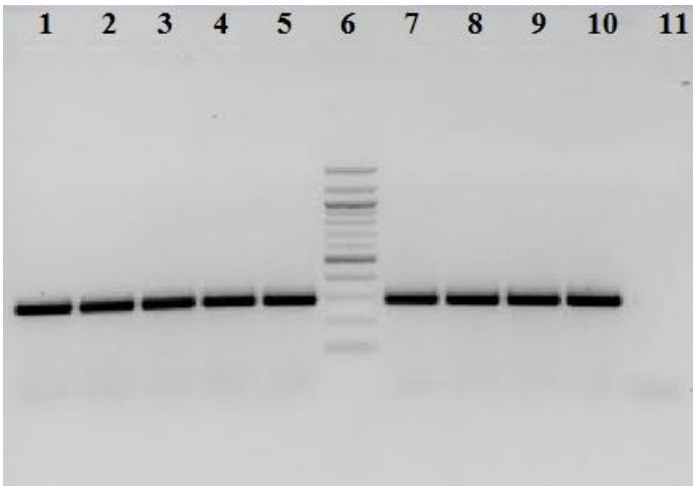


Figure 2.14. *Enterococcus sodA* gene using Pigmented/Motile isolates presumptively *E. casseliflavus*. Lane 1, 8pm 22; lane 2, 8pm 24; lane 3, 8pm 39; lane 4, 8pm 40; lane 5, 8pm 37; lane 6, 100bp molecular marker (New England Biolabs); lane 7, 8pm 23; lane 8, 8pm 25; lane 9, 8pm 28; lane10, *E. casseliflavus* ATCC 51328; lane 11, negative control (No DNA added).

2.3.4.2 Non pigmented-non motile isolates

Considering the importance of the non-pigmented pathogenic species such as *E. faecalis* and *E. faecium*, we evaluated the effectiveness of the *atpA* marker for their identification. Both species, *E. faecalis* and *E. faecium*, presented the 1,102-bp *atpA* amplification. In order to validate the method clinical isolates presumptively identified as *E. faecalis* and *E. faecium* using the Vitek 2 system (bioMérieux) were also analyzed. The RFLP analysis of *atpA* gene from these strains (Figure 2.15) revealed that each species had its own band pattern, with some characteristics distinctive bands for each one. As a highlight, in Figure 2.15-A (lane 7), there is not a lack or blurred bands that may confuse the identifying of *E. faecalis* as expected. Otherwise, in Figure 2.15-B, we observed one band pattern (lane 7) totally different in comparison with the other results for *E. faecium*, including the positive control (lane 8).

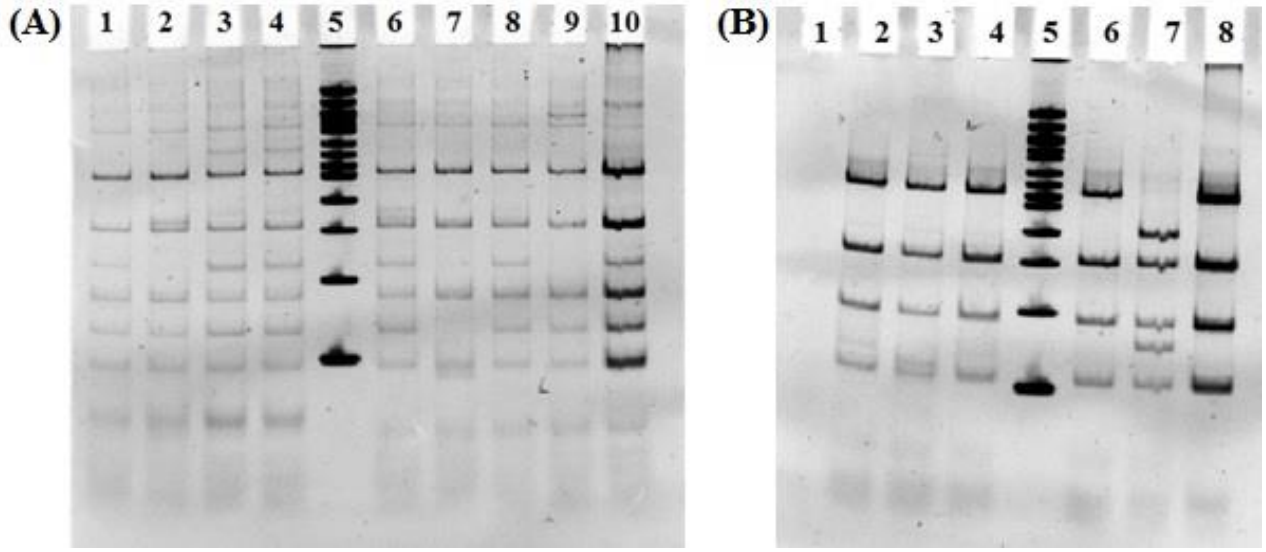


Figure 2.15. *Enterococcus atpA* gene RFLP. **(A)** Presumptively *E. faecalis*: Lane 1, B12-2379; lane 2, B12-2684-2; lane 3, 12-3526; lane 4, 12-3586-2; lane 5, 100bp molecular marker (Promega); lane 6, 12-3622; lane 7, 12-3656; lane 8, 12-3769; lane 9, 12-3643; lane10, *E. faecalis* ATCC 19433. **(B)** Presumptively *E. faecium*: Lane 1, Empty; lane 2, 12-3863; lane 3, 12-4955; lane 4, 12-4991; lane 5, 100bp molecular marker (Promega); lane 6, 12-4291; lane 7, 12-4115; lane 8, *E. faecium* ATCC 19434.

These points led us to confirm the speciation using additional presumptively *E. faecalis* and *E. faecium* isolates. In this case, the samples were provided by Donna Ferguson (Southern California Coastal Water Research Project and University of Los Angeles, California), who also used the Vitek 2 (bioMérieux) system to identify them. The possible misinterpretation by blurred band patterns between the *E. faecalis* results was clarified when we compared these patterns one next to the other (Figure 2.16; lanes 1-6). Using the 100-bp DNA marker (lane 7) as a reference, the main band pattern labeled as *E. faecalis* was: **90-bp, 120-bp, 180-bp, 500-bp and 600-bp**. These results also demonstrated that *E. faecium* has two band patterns: (a) **110-bp, 180-bp, 500-bp and 700-bp** (lane 8) or (b) **150-bp, 210-bp, 250-bp, 505-bp and 600-bp** (lanes 9 and 10).

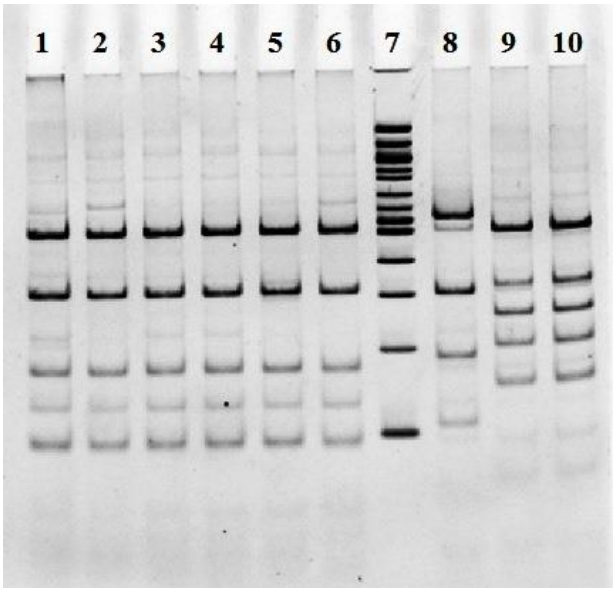


Figure 2.16. *E. faecalis* and *E. faecium atpA* gene RFLP. Lane 1, D11; lane 2, D12; lane 3, D15; lane 4, D24; lane 5, D28; lane 6, D29; lane 7, 100bp molecular marker (Promega); lane 8, D60; lane 9, D62; lane10, D63.

2.3.5 Validation of the method

In order to validate these results of the band patterns as *E. faecalis* and *E. faecium* species identification, *sodA* gene Multiplex PCR published by Jackson et al. (2004) was also used. This method amplifies a species-specific amplicon of 360-bp for *E. faecalis* and 215-bp for *E. faecium* respectively. Figure 2.17 presents the results confirming these two species previously identified by the *atpA* RFLP method of these two species previously identified. DNA in lanes 1-2 belonged to the *E. faecalis* speciation that matched perfectly with the positive control (lane 3) and, also, our RFLP identification results (Figure 2.15-A; lanes 6-7). Moreover, considering the two apparent *E. faecium* RFLP results, lanes 5-6 (Figure 2.17) corresponded to the first pattern (Figure 2.15-B; lanes 3-4) and the lanes 9-10 (Figure 2.17) to the second one (Figure 2.16; lanes 9-10). Despite the notable differences between these two *atpA* RFLP patterns, both were confirmed to be *E. faecium* through the expected 215-bp *sodA* fragment amplification, including the positive controls (Figure

2.17; lanes 7 and 11). Using this information and the 100-bp DNA marker as a molecular weight reference, we officially described the *E. faecalis* band pattern as: **90-bp, 120-bp, 180-bp, 500-bp and 600-bp**. In the same way, the two possible *E. faecium* band patterns are: (a) **110-bp, 180-bp, 500-bp and 700-bp** or (b) **150-bp, 210-bp, 250-bp, 505-bp and 600-bp**.

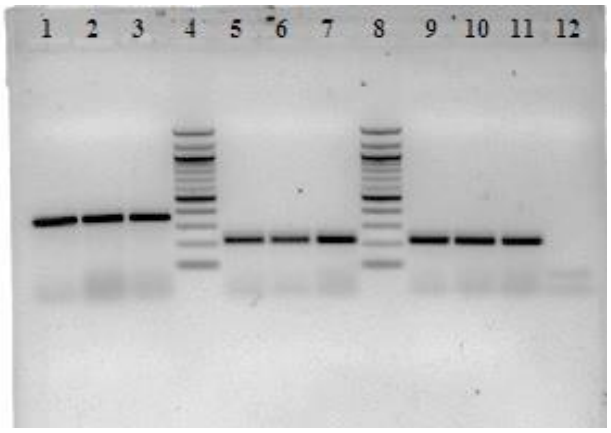


Figure 2.17. *E. faecalis* and *E. faecium sodA* gene Multiplex PCR (Group 1). Lane 1, 12-3622; lane 2, 12-3656; lane 3, *E. faecalis* ATCC 19433; lane 4, 100bp molecular marker (Promega); lane 5, 12-4955; lane 6, 12-4991; lane 7, *E. faecium* ATCC 19434; lane 8, 100bp molecular marker (Promega); lane 9, D62; lane10, D63; lane 11, *E. faecium* ATCC 19434; lane 12, Negative control (No DNA added).

Overall, we used our collection of enterococci controls to validate the speciation through a comparison between the *sodA* gene Multiplex PCR and the *atpA* RFLP method. Results in Figure 2.18 demonstrate that the corresponding *sodA* gen fragment was amplified for each species. Moreover, once the *atpA* RFLP patterns of these enterococci species (Figure 2.19) were unique for each of them.

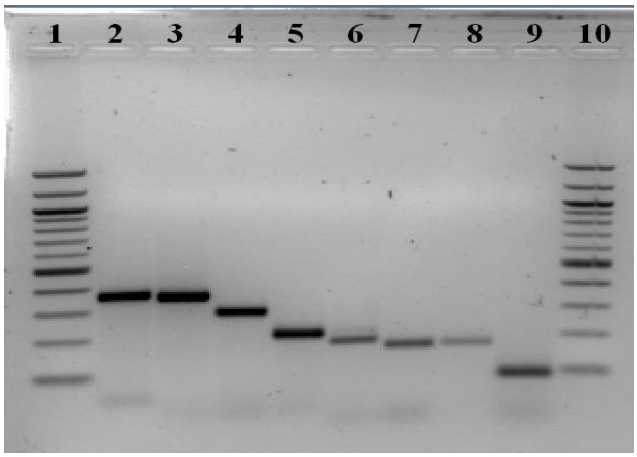


Figure 2.18. Jackson PCR (*sodA* gene) using enterococci positive controls. Lane 1, 100bp molecular marker; lane 2, *Enterococcus avium* (368bp); lane 3, *Enterococcus faecalis* (360bp); lane 4, *Enterococcus durans* (295bp); lane 5, *Enterococcus faecium* (215bp); lane 6, *Enterococcus gallinarum* (173bp); lane 7, *Enterococcus pallens* sample; lane 8, *Enterococcus pallens* (160bp); lane 9, *Enterococcus mundtii* (98bp); lane 10, 100bp molecular marker.

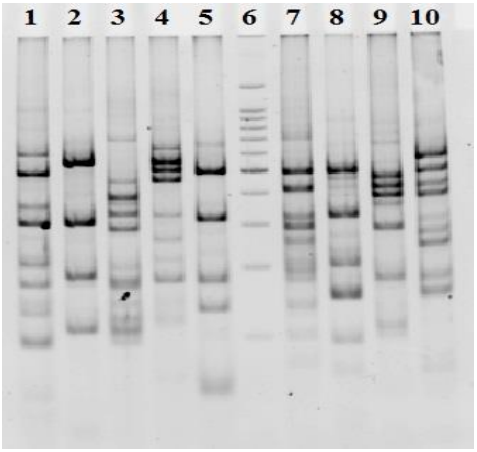


Figure 2.19. *atpA* gene RFLP using enterococci positive controls. Lane 1, *Enterococcus faecalis*; lane 2, *Enterococcus faecium;* line 3, *Enterococcus avium*; lane 4, *Enterococcus gallinarum;* lane 5, *Enterococcus aquimarinus;* lane 6, 100bp molecular marker; lane 7, *Enterococcus casseliflavus*; lane 8, *Enterococcus mundtii*; lane 9, *Enterococcus pallens*; lane 10, *Enterococcus gilvus.*

2.4 Discussion and conclusions

Although all enterococci are not necessarily pathogenic, more than 104 CFU/100ml in a single sample are considered as an alert of fecal pollution that might result in an increase of health risks. However, considering the abundance and diversity of species that frequently are found on fecal matter, we believe that a simple enumeration might not be a reliable association between this guideline and health implications. During the 1980s, studies demonstrated that the number of fecal indicator bacteria was associated with an increase in the probability of swimmers to become ill (Cabelli et al., 1983; Dufour et al., 1984). However, although recently epidemiological studies in recreational waters supported this presumption using modern quantitative molecular analyses such as qPCR (Sinigalliano et al., 2010 ;Wade et al., 2010) and QMRA (Soller et al., 2010), none of them identified the enterococci species or distribution.

Even more, the few studies that reported the enterococci species distribution in recreational waters used identification methods that were developed from the clinical perspective such as the API 20 Strep rapid kit (Bio-Merieux; Ferguson et al., 2005), or MicroScan system (Siemens Healthcare; Moore et al., 2008) that lacks in high misidentification (Angeletti et al., 2001) and discrepancies among environmental enterococci species (Moore et al., 2006).

It is well known that gram positive cocci that have the capacity to grow in 6.5% NaCl and at 45°C, hydrolyze esculin and are catalase-negative should be members of *Enterococcus* (Devriese et al., 1992; Manero et al., 1999). However, considering the time required doing all these tests, a rapid molecular-based method was developed to identify *Enterococcus* to the genus level using the amplification of a 112-bp fragment of the *tuf* gene (Ke et al., 1999). However, our results showed that a pure culture of *Staphylococcus aureus* also amplifies producing a PCR fragment of equal size as the expected for enterococci (Figure 2.2). *Staphylococcus* could also grow on the selective mE media also (Eaton et al., 2005); therefore, we used the catalase reaction to eliminate any *Staphylococcus* spp. from our study.

To date, the molecular-based identification methods are considered to be the most reliable, but most of them require sequencing to have accurate results. Actually, some of them have a low potential to discriminate species within the same group (Patel et al., 1998). Furthermore, PCR-based methods that have been performed, such as: ITS-PCR (Tyrrell et al., 1997), AFLP typing (Vos et al., 1995) and REP-PCR (Versalovic et al., 1991), had discrepancies within the environmental enterococci species (Pangallo et al., 2008) and other methods require multiple mixture reactions (Jackson et al., 2004). As a more rapid alternative, we proposed a RFLP method using the amplification of the *atpA* gene followed by a double digestion and gel electrophoresis instead of sequencing as originally published (Naser et al., 2005).

The practical functionality of the *atpA* RFLP method was evaluated *In silico* test considering it as a tool that could help us to improve it prior the practical experience. However, in contrast with the *In silico* results that demonstrated the recovery of the 1,102-bp *atpA* fragment and a good quality of the digestion, we observed several limitations in the practical results such as: non-amplification, blurred bands and unspecific results in our practical experience. Better *atpA* RFLP amplifications were observed using the chemical lysis to extract the DNA and the Promega Flexi Taq Polymerase with the pigmented-motile strains. Furthermore, these strains, were also confirmed as *E. casseliflavus* using the *sodA* 288-bp fragment amplification (Figure 2.14) considering they presented different *atpA* RFLP band patterns (Figure 2.13). Although there is no evidence, preliminary explanations about the inconsistency of the band patterns recovered with *E. casseliflavus* could be: low conservation of the *atpA* gene, unspecificity of the primers and multiple priming sites within the *E. casseliflavus atpA* fragment and/or the whole genome are responsible for our observations.

Even though our method has a limitation identifying *E. casseliflavus* isolates, more than 378 enterococci isolates, including ATCC controls, were identified with our method. It should be noted that consistently three enterococci species were identified satisfactorily: *E. faecalis*, *E. faecium* and *E. gallinarum*. In addition to the non-motility characteristic, *E. faecalis* can be identifiable by a unique specific *atpA* RFLP pattern, in contrast to *E. faecium* that had two distinct patterns (Figures 2.15 and 2.16). There are two patterns that represented *E. faecium*, 76% (*n*=125) of the isolates displayed one pattern (110-bp, 180-bp, 500-bp and 700-bp) and the rest of the isolates the other pattern (150-bp, 210-bp, 250-bp, 505-bp and 600-bp). Also, these patterns (the one for *E. faecalis* and the two for *E. faecium*) were confirmed using the *sodA* 360-bp and 215-bp species-specific fragments amplifications, respectively (Figure 2.17). Besides the capacity to

identify *E. faecalis* and *E. faecium*, we were also able to recognize the characteristic pattern of *E. gallinarum* (Figure 2.19), although it was recovered only in 2% of the isolates.

The effectiveness of the *atpA* RFLP method was evaluated with the *sodA* Multiplex PCR developed by Jackson et al., 2004. Despite the disadvantage of time-consuming as it request the set-up of seven reactions, this was a useful method that helped us to confirm our speciation results (Figures 2.18 and 2.19). Although these methods were efficient reproducible with respect to the identification of *E. faecalis, E. faecium and E. gallinarum*, we can't validate it with the rest of the species considering that weren't typically found in the environment.

In view of the consistent abundance of specific enterococci species in the environment, in contrast with the diversity reported on human and animal fecal matter (Layton et al., 2010), plants (Müller et al., 2001) and soil (Micallef et al., 2013), we suggest that in addition to a numerical analysis also species distribution could also be important as indicative of potential "fecal pollution" and/or illness risks. Considering there is no data available about this aspect, further investigation concerning the distribution and behavior of enterococci community through a 24 hours study in the environment is presented in the next part (chapter 3).

Chapter Three

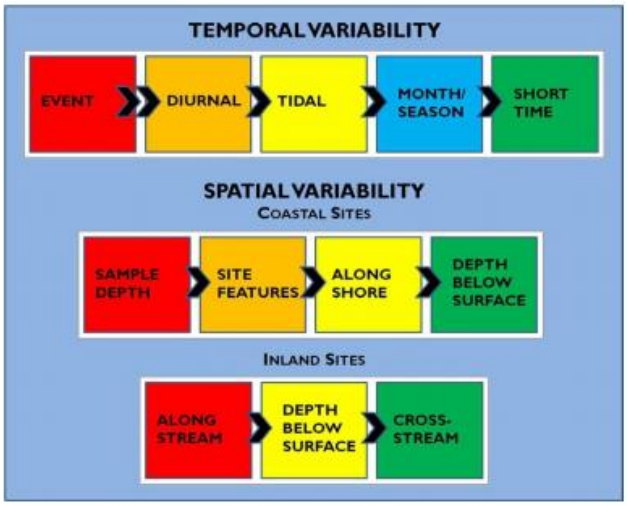
Enumeration and monitoring enterococci community in a seawater system during a 24-hour period

3.1 Introduction

The United States Environmental Protection Agency (USEPA, 1986) established guidelines to monitor recreational water quality using concentrations of fecal indicator bacteria (FIB) that included total coliforms, *E. coli*, and enterococci. Although the USEPA uses three fecal indicator bacteria, the enterococci are considered the best indicator of pollution in seawater (Hanes & Fragala, 1967; Noble et al., 2003). The enterococci guidelines established that a beach is contaminated when the concentrations exceed the standard of 104 CFU/100ml in a single sample analysis (USEPA, 1986). Additionally, the USEPA also established in these guidelines a method of multiple samples quantified the geometric mean of 35 CFU/100ml. However, this geometric mean guideline was revised in 2012 and new recommendations were added as follows: (i) a secondary geometric mean parameter and (ii) the addition of a statistical threshold value (STV) for each one. Specifically, the original standard of 35 CFU/100ml was complemented by a STV of 130CFU/100ml; in addition, the establishment of a new guideline of 30CFU/100ml with a STV of 110CFU/100ml.

Actually, it is considered that a beach is unsafe for swimmers when the FIB exceeds those numerical standards. In addition, the USEPA issued further considerations to monitoring recreational waters by culture methods in 2010. The purpose of this new modifications emphasized the influence of factors that could affect the density of FIB during the sampling, such as the temporal and spatial variability as the most important points to be considered (Exhibit 1, USEPA 2010) prior to sampling.

Exhibit 1. Ranking of factors influencing variability of fecal indicators in natural systems



Note: Temporal variability at a short time scale is ranked lowest, except for samples obtained at ankle depth and shallower.

Within the temporal variability, the diurnal sampling is categorized as the greatest important factor. Morning sampling was recommended to find the highest density of fecal indicator bacteria in recreational waters that may point to human-influenced events (e.g., treated waste water effluent discharges) (USEPA, 2010). In contrast, previous studies have found that the highest values of fecal indicators occur during night hours (Boehm, 2007; Rosenfeld et al., 2006). Sunlight inactivation has been proposed as the major contributor of the density decrease during the day (Sinton et al., 2002; Liu et. al., 2006; Whitman et al., 2004).

Although it is known that these enterococci guidelines were established in non-tropical waters, the subtropical environment of which may promote a different behavior of the FIB being monitored. However, considering the frequent exceedance of the enterococci guidelines in tropical environments (Shibata et al., 2004) and, previous studies that demonstrate the presence of enterococci in subtropical non-point source beach (Abdelzaher et al., 2010; Fleisher et al., 2010), it could be questioned the reliability of this indicator as a good tropical fecal pollution indicator.

Furthermore, other factors such as the natural occurrence or persistence of enterococci in soils, sediments, and aquatic vegetation have been proposed as factor contributing to the high concentration of enterococci (Fujioka et al., 1998; Anderson et al., 2005; Badgley et al., 2010). The probability of re-suspension in the water by the tidal influence has been also proposed (Desmarais et al., 2002; Shibata et al., 2004).

Although previous works have reported the occurrence and prevalence of some enterococci species in human and animal fecal matter (Layton et al., 2010), plants (Müller et al., 2001) and soil (Micallef et al., 2013), little is known about background levels and the diversity of enterococci species in the marine environment when the samples are within the established parameters (Ferguson et al., 2005; Moore et al., 2008). However, considering the variability and the occurrence based on the enterococci species found in different sources (feces, septic tanks, WWTP, runoff), the aim of this study was to monitor the enterococci community in a coastal marine water system every 4 hours for a 24-hour period.

We hypothesized that the USEPA 1600 method does not describe a recent fecal contamination event in subtropical marine waters.

3.2 Materials and methods

3.2.1 Water sample collection

To evaluate changes in the concentrations of the enterococci community during a 24-hour period, water samples were taken and processed in 4-hour intervals. The sampling site was at the Mani's beach at Mayagüez (18.232132°, 67.172740°; Google Earth). The beach was chosen for

the easy access, convenient distance from our laboratory, and the relatively limited access to the public. Also, this beach has no direct impact from rivers or recreational bathers having the potential of being a site mainly non-point source of contamination.

The sampling occurred during the year (2009 to 2012), within dry (December to April) and wet seasons (May to November), as determined by the new moon phase to avoid tidal differences. The specific sampling dates were on: December 20-21, 2009; March 14-15, 2010; May 15-16, 2010; June 11-12, 2010 and August 18-19, 2012. As external factors that may affect our results, weather conditions such as rain and storms were considered using weather predictions prior to the sampling. The sampling started at 12:00 am (sample ID: 12:00 am I) and ended at the same time the following day (sample ID: 12:00 am II) for a total of seven samples that were taken in triplicate (3 individual bottles) every 4 hours. Each sample consisted of 100 ml that was collected approximately 1 m from shore, at ankle/knee depth, in sterile wide mouth 250 ml bottles. To evaluate the aseptic techniques, we used a field blank of 100 ml sterilized distilled water that was opened during sampling. The samples were transported on ice and processed 1hr after collection.

3.2.2 Sample processing

Samples were submitted to the USEPA Method 1600 (USEPA, 2000) using mEnterococcus agar (mE), instead of mEI, and incubated for 48hrs at 41°C (Precision; Thermo Scientific). Dark red to maroon colonies were interpreted as presumptive enterococci. Results were expressed as the average of the triplicates enumerated, with their corresponding standard deviation, as colony forming units (CFU) per 100 ml of water.

3.2.3 Biochemical tests

In order to confirm the enumerated isolates as enterococci, at least 10% (depending on total numbers) of the colonies, per sample, were randomly chosen from the three filters with sterile wooden toothpicks and transferred to Brain Heart Infusion Agar. Biochemical tests included: catalase reaction in 3% hydrogen peroxide, esculin hydrolysis in Bile Esculin Agar, motility in Sulfate Indol Motility medium and growth in 6.5% NaCl and at 45°C in Brain Heart Infusion Broth. All isolates that were catalase negative and had positive reactions for esculin hydrolysis, and grew in 6.5% NaCl at 45˚C were identified as *Enterococcus* species.

3.2.4 Enterococci groups

Confirmed enterococci isolates were separated into four groups based on their pigmentation and motility in an attempt to identify them to the species level (Figure 3.1) and determine changes in the dominance and distribution of the community.

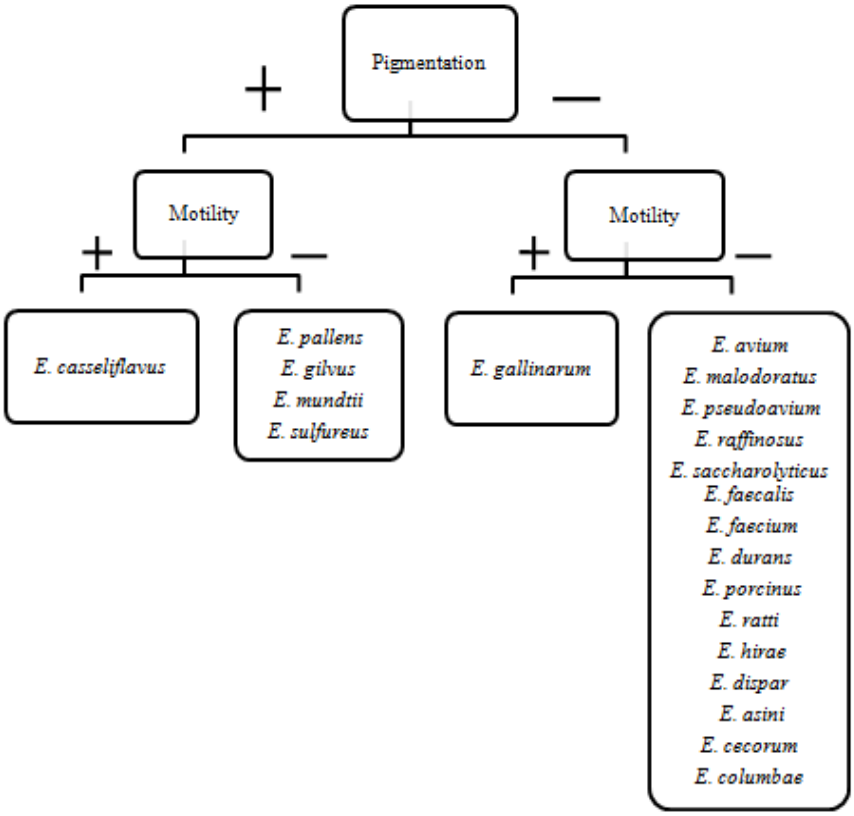


Figure 3.1. Grouping of some enterococci species by pigmentation and motility characteristics.

3.2.5. Species identification

Enterococci isolates were molecularly identified to the species level using the methodology presented in Chapter two.

3.2.6. Tidal levels

In order to evaluate a relationship between the tide levels and the enterococci average concentrations, we used the public available data for the Mayagüez region published by the National Oceanic and Atmospheric Administration of United States (NOAA/ NOS/ Center for Operational Oceanographic Products and Services). We labeled the range of the tide level data as "low" from 0 to 0.5 feet and as "high" for values above 0.5 feet.

3.2.7. Multiplex PCR of virulence factors

We amplified the DNA samples to detect the presence of virulence factors using a Multiplex PCR method previously described (Vankerckhoven et al., 2004) with the modification of using the Promega Flexi Taq DNA polymerase instead the Hot-StarTaq DNA polymerase. Each set of primers had a characteristic product size to differentiate within the five virulence genes (*asa1* at 375bp, *gelE* at 213bp, *cylA* at 688bp, *esp* at 510bp and *hyl* at 276bp). In addition, we used DNA from the *E. faecalis* strain MMH594 (provided by Dr. Nathan Shankar) as a positive control carrying four of the five virulence genes (*asa1*, *gelE*, *cylA*, and *esp*). No positive control was available to confirm the presence of hyaluronidase (*hyl*). All PCR product sizes were confirmed by 1.8% agarose-gel electrophoresis at 110v / 90min in 1X Tris-Acetate-EDTA (TAE Buffer; 8.0pH), stained with ethidium bromide, and visualized by UV transillumination. We documented the gel results using a molecular imager system (VersaDoc MP 4000).

3.3 Results

3.3.1. Enumeration of enterococci

After the 48-hour incubation time in selective mE media, the enterococci isolates were enumerated as Colony Forming Units per 100mL (CFU/100mL). The average and standard deviation were calculated using the three replicates taken at each of the sampling intervals. The sampling months were at December (2009), March (2010), May (2010), June (2010) and August (2012).

The results demonstrated that the average enumeration of enterococci does not exceed the USEPA guideline at the same hour or period of the day. The exceedance events in March (Table 3.2), June (Table 3.4) and August (Table 3.5) were at night whereas, during May (Table 3.3) these

occurred during the day, and in December (Table 3.1) during the day and night.

On the other hand, considering the standard deviation, high values were predominantly observed while high enumerations occurred; however, this was not true for all the sampling points / months. For instance, in December (Table 3.1), the standard deviations values were high for most of the results, regardless of the average enumerations. Furthermore, on this month at 12:00 am the standard deviation range overlapped the USEPA guideline demonstrating that one of the triplicate samples exceeded the limit, even though the average enumeration did not.

Table 3.1. Average enumeration of enterococci on December 2009.

Sampling Hours	Enterococci Average Enumeration (CFU/00mL)	Standard Deviation (±)
12:00am	101	38
4:00am	64	10
12:00pm	119	41
4:00pm	6	4
8:00pm	42	24
12:00am	225	24

Otherwise, considering the results of March (Table 3.2), May (Table 3.3) and June (Table 3.4), we observed similar consistent high standard deviations values while high average enumerations were achieved, especially during the exceedance events. This proportional relationship was also observed within the low values, this is, low standard deviations while low enumerations.

Table 3.2. Average enumeration of enterococci on March (2010).

Sampling Hours	Enterococci Average Enumeration (CFU/00mL)	Standard Deviation (±)
12:00am	52	11
4:00am	48	27
8:00pm	42	14
12:00pm	56	7
4:00pm	17	5
8:00pm	358	64
12:00am	11	3

Table 3.3. Average enumeration of enterococci on May (2010).

Sampling Hours	Enterococci Average Enumeration (CFU/00mL)	Standard Deviation (±)
12:00am	64	7
8:00pm	31	5
12:00pm	35	8
4:00pm	161	40
8:00pm	44	8
12:00am	60	15

Table 3.4. Average enumeration of enterococci on June (2010).

Sampling Hours	Enterococci Average Enumeration (CFU/00mL)	Standard Deviation (±)
12:00am	144	38
4:00am	30	3
8:00pm	71	10
12:00pm	3	2
4:00pm	12	6
8:00pm	4	2
12:00am	469	55

However, in August 2012 (Table 3.5), despite the increase of the enterococci densities during the two exceedance events, the standard deviations were consistent values. Even more, in contrast with the previous months, we could not determine any pattern or relationship between the values in this month considering that in most of the sampling hours the standard deviation varied regardless the average enumeration.

Table 3.5. Average enumeration of enterococci on August (2012).

Sampling Hours	Enterococci Average Enumeration (CFU/00mL)	Standard Deviation (±)
12:00am	15	17
4:00am	41	8
8:00pm	25	22
12:00pm	12	11
4:00pm	42	5
8:00pm	183	26
12:00am	258	25

As an overview of the results, the Figure 3.2 shown the enumeration results recovered in the whole 24-hour study months. In total, using as reference the 104CFU/100ml USEPA guideline (red line), eight (8) exceedance events were observed: one (1) at 12:00pm (December; blue line), one (1) at 4:00pm (May; purple line), two (2) at 8:00pm (March; green line, August; gray line) and four (4) at 12:00am (December; blue line, August; gray line and June; pink line). However, none exceedance events were found at 8:00am.

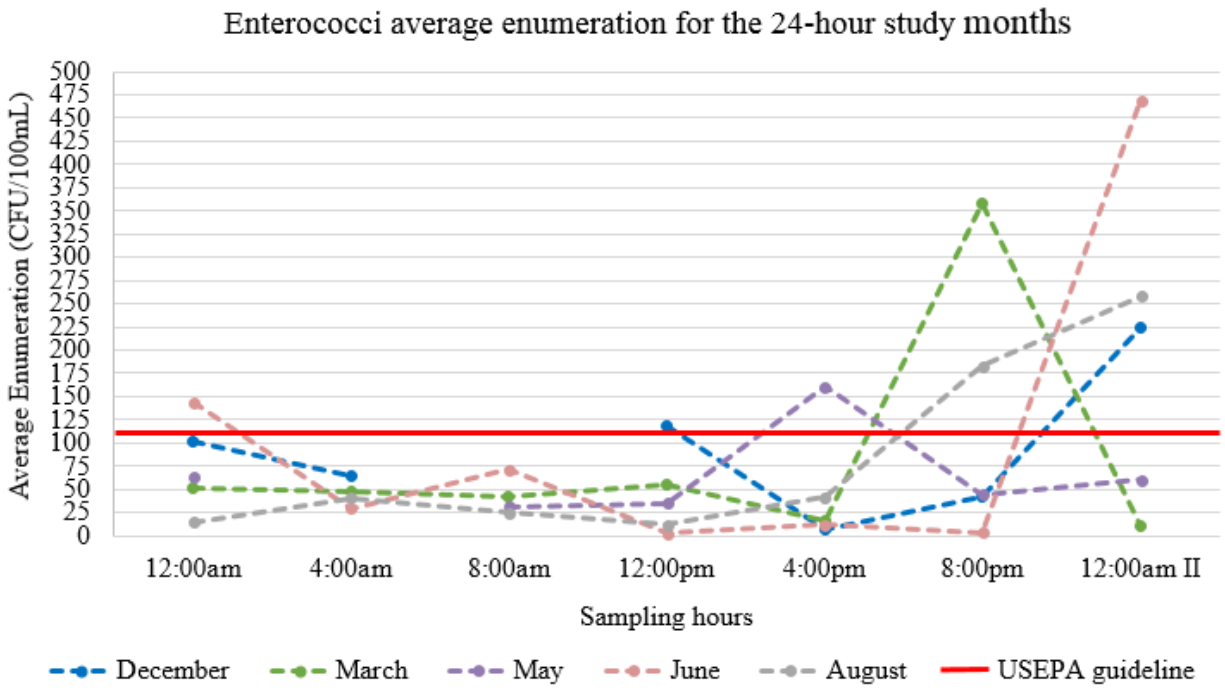


Figure 3.2. Enterococci average enumeration for the five months of the 24-hour study: December, 2009 (blue line); March, 2010 (green line); May, 2010 (purple line); June, 2010 (pink line); and August, 2012 (gray line) using the 104CFU/100ml USEPA guideline (red line) as the limit reference.

3.3.2 Geometric Mean and STV guidelines

The previously presented data suggests that the water quality is contaminated using the numerical USEPA enterococci guideline of 104CFU/100ml at least at certain times during the day. In order to confirm this, the alternative analysis of the geometric mean for multiple samples (USEPA, 2012) was used. This method establishes the following recommendations:

1: “Concentrations of enterococci bacteria shall not exceed 35 CFU/100 ml based upon the geometric mean of samples taken in any thirty (30) days interval and the statistical threshold value (STV) of 130 CFU/100 ml should not be exceeded by more than 10 percent of the samples taken during the same thirty (30) days interval.”

or

2: “Concentrations of enterococci bacteria shall not exceed 30 CFU/100ml based upon the geometric mean of samples taken in any thirty (30) days interval and the statistical threshold value (STV) of 110 CFU/100ml should not be exceeded by more than 10 percent of the samples taken during the same thirty (30) days interval.”

These two alternatives were evaluated to determine if the results of water quality change through the guidelines for single and multiple samples. For this, we used the three replicates enumeration, instead of the average, per sampling hours resulting in, at least, 21 individual samples per each study month.

The overall results are presented in the Table 3.6, show that regardless of the alternative recommendation used none of the study months comply with any of these USEPA guidelines. Specifically, during December, March and August, neither the geometric mean nor the percent samples/STV complies with the established limit standards. However, although in May a difference was observed among the recommendations in the percent of samples / STV values still exceeded the standards. Likewise, in June the calculated geometric mean value of 28CFU/100mL was within the limits of the two parameters, this did not occur for the percent samples/STV values, that mean a non-compliance of the standard.

Table 3.6. Comparison of the geometric mean (GM) and statistical threshold value (STV) using the recommendations 1 (red) and 2 (blue) issued by the USEPA (2012) revised criteria.

Month	Recommendation 1		Recommendation 2	
	Calculated GM (CFU/10mL)	Calculated STV (% of samples)	Calculated GM (CFU/10mL)	Calculated STV (% of samples)
December	56	28%	56	28%
March	43	14%	43	14%
May	55	11%	55	18%
June	28	24%	28	24%
August	49	29%	49	29%

3.3.3 Tidal levels

During December (Figure 3.3), the enterococci enumeration showed an inverse relationship with the tide levels. Specifically, at 4:00pm, we recorded the lowest enterococci enumeration (6CFU/100ml) while the tide reached the highest level (1.5ft). Similarly, at 12:00am II, we got the highest enumeration of enterococci (225CFU/100ml) while the tide level was at the lowest (0.4ft.). In fact, this behavior was observed in the rest of the sampling points regardless of the month (Figures 3.3 to 3.7).

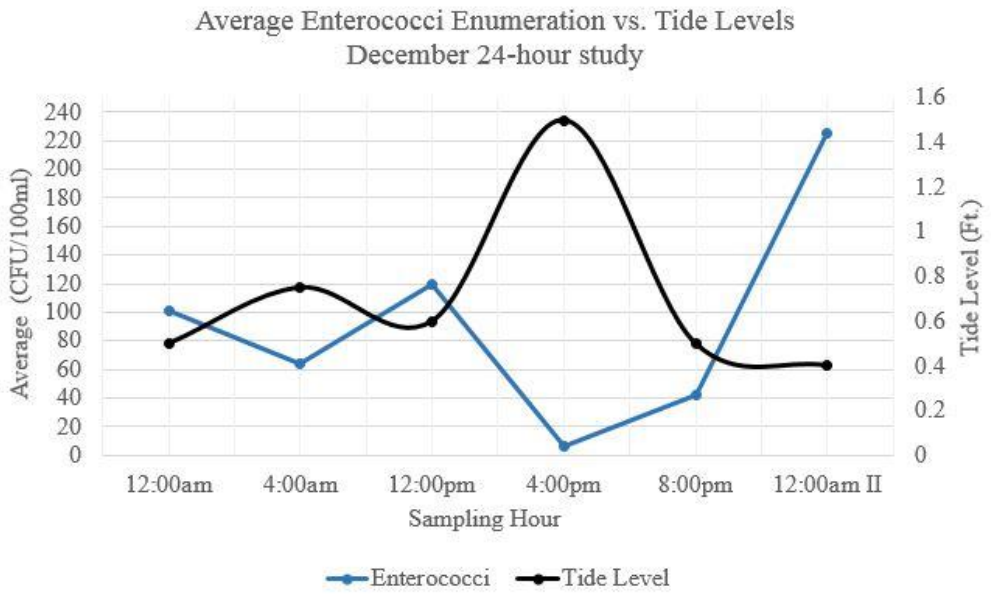


Figure 3.3. Tide levels and average enterococci enumeration at Mayagüez, P.R on December 20-21, 2009.

In March (Figure 3.4), low enterococci concentrations (under permissible levels) were consistently enumerated from 12:00am to 4:00pm, even though the tide levels varied from low to high during this period. Otherwise, at 8:00pm, the enterococci reached the highest result (358CFU/100ml) even when the tide level relatively stayed at the low value of 0.3ft. In contrast, no specific relation between tide levels and enterococci counts was observed for the month of May (Figure 3.5).

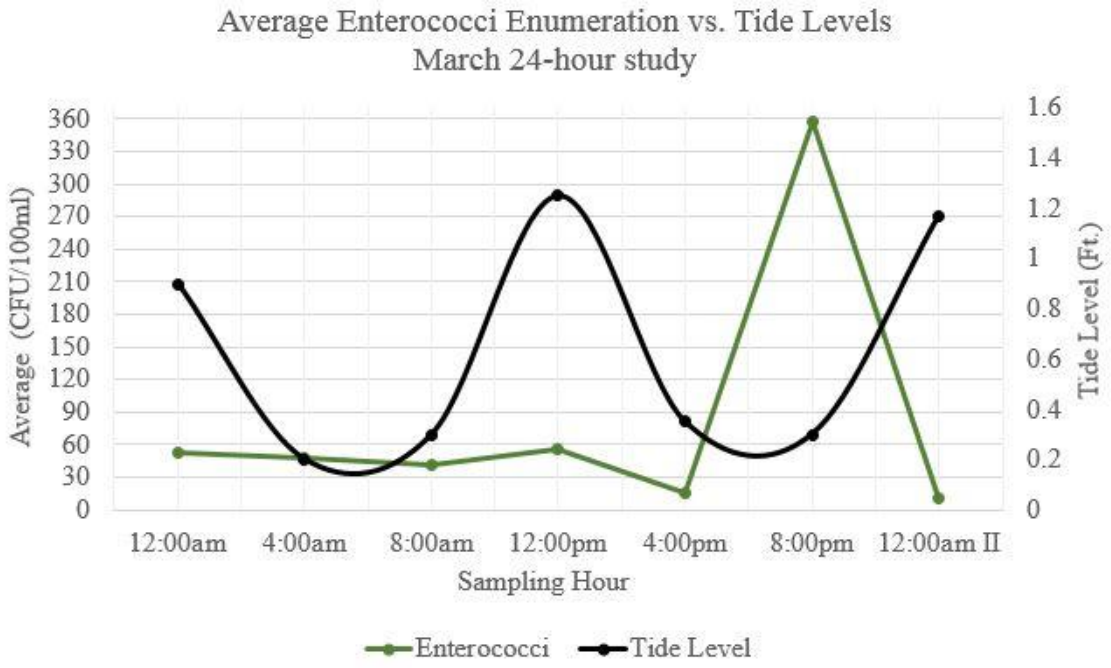


Figure 3.4. Tide levels and average enterococci enumeration at Mayagüez, P.R on March 14-15, 2010.

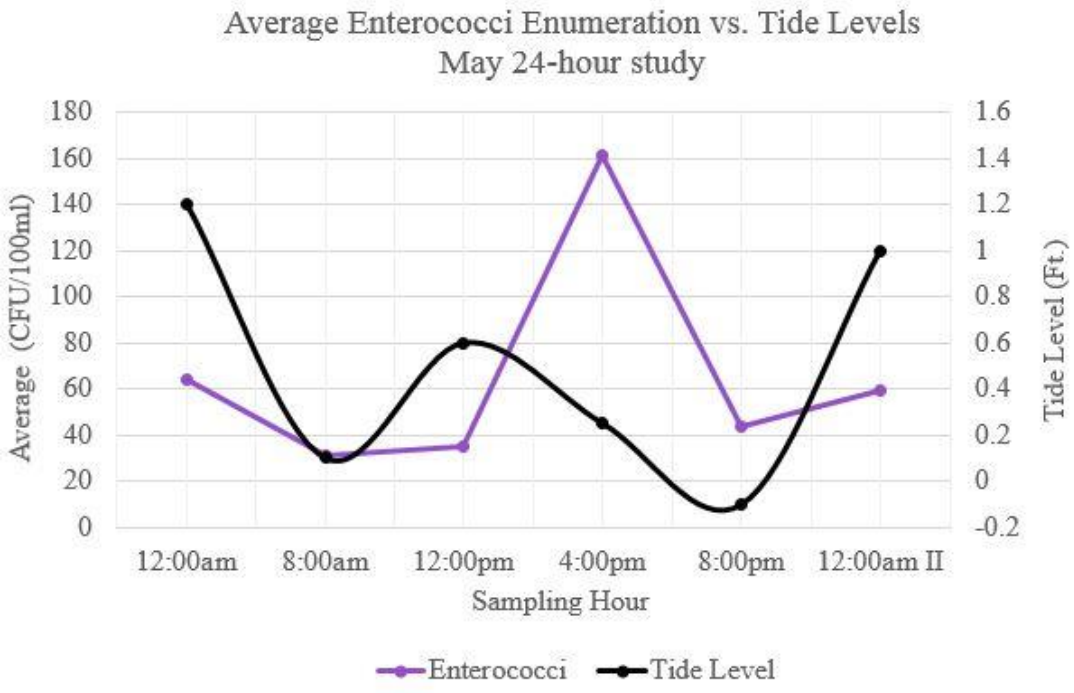


Figure 3.5. Tide levels and average enterococci enumeration at Mayagüez, P.R on May 15-16, 2010.

Furthermore, considering the results of the study in June (Figure 3.6), both variables had a similar behavior with a parallel decreasing from 12:00am until 4:00am and, in the same way, an increasing from 8:00pm until 12:00am II. However, at the middle of the study period, specifically from 8:00am until 4:00pm, an inverse decreasing/increasing relationship had been detected.

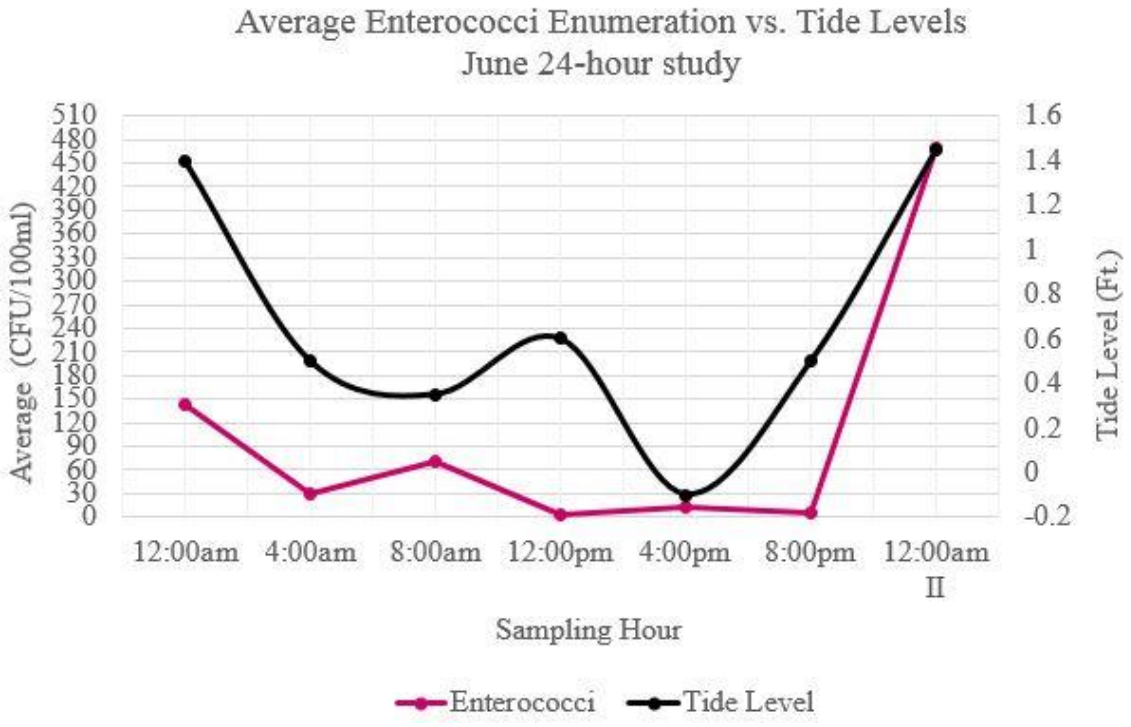


Figure 3.6. Tide levels and average enterococci enumeration at Mayagüez, P.R on June 11-12, 2010.

Finally, in August (Figure 3.7) we observed a similar relationship as the one described earlier for the month of March (Figure 3.8). Specifically, from 12:00am until 4:00pm low enterococci concentrations were consistently found, even regardless of the tide levels during this time period. However, in this month the enterococci densities reached the highest value during high tide level in the last sampling point.

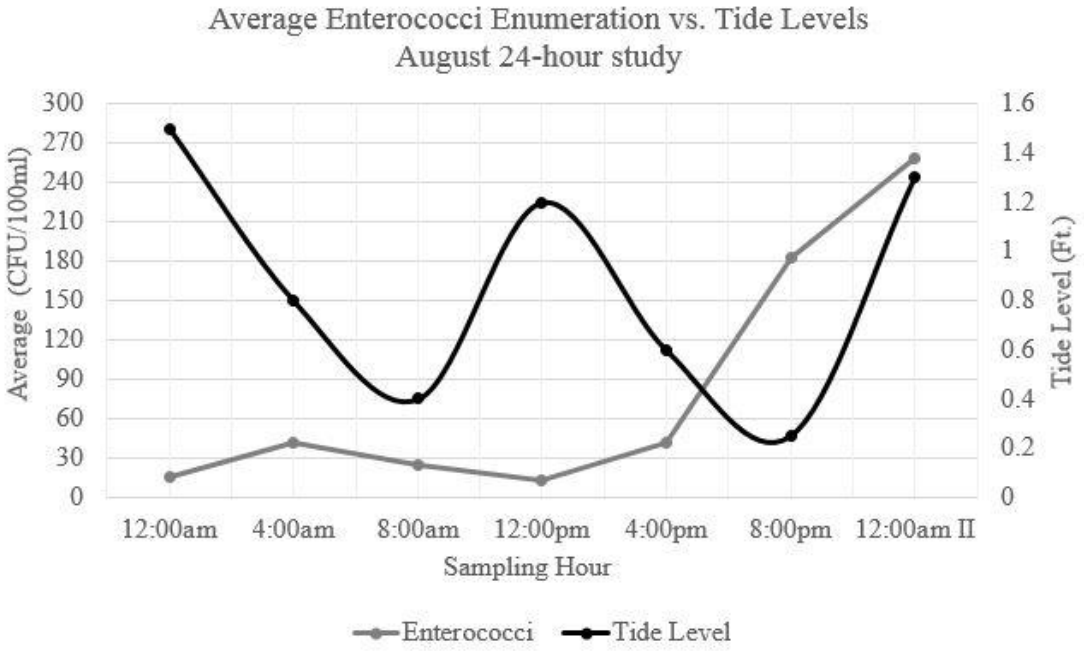


Figure 3.7. Tide levels and average enterococci enumeration at Mayagüez, P.R on August 18-19, 2012.

3.3.4. Distribution of enterococci groups

During the month of December, 25% (139 isolates) of the 557CFU/100ml enumerated enterococci were evaluated (Table 3.7). Consistent changes in the community were observed in the occurrence (% of the groups) and dominance (the highest % of one group relative to others). Three groups dominated the community at different times: (i) pigmented-non motile (12:00am and 4:00am), (ii) pigmented-motile (12:00pm) and (iii) non pigmented- non motile (4:00pm, 8:00pm and 12:00am II). Interestingly, although we recorded two exceedance events, at 12:00pm and 12:00am II, each of them was dominated by a different group, pigmented and non-pigmented, respectively. The first exceedance event was followed by a community reduction of 95% from, 119 to 6 CFU, four hours later (4:00pm) and, more importantly, the dominant enterococci changed from pigmented to non-pigmented. Furthermore, using this approach we were able to show that the yellow pigmented (motile or not) were the dominant group in December, with 54% of the

community, followed by the non-pigmented (46%). More importantly, the diversity of the enterococci community changed at least every 4 hours with or without an increase of the total number of enterococci.

Table 3.7. Pigmentation and motility groups of the analyzed enterococci in December (2009).

Sampling hours	Total Enumeration (CFU/100ml)	Analyzed isolates *n* (%)	Pigmentation and motility groups			
			Pigmented / motile *n* (%)	Pigmented / non motile *n* (%)	Non pigmented / motile *n* (%)	Non pigmented / non motile *n* (%)
12:00am	101	33 (33)	8 (24)	13 (39)	1 (3)	11 (33)
4:00am	64	17 (27)	4 (24)	8 (47)	1 (6)	4 (24)
12:00pm	119	25 (21)	16 (64)	2 (8)	1 (4)	7 (28)
4:00pm	6	6 (100)	0 (0)	2 (33)	1 (17)	3 (50)
8:00pm	42	18 (43)	2 (11)	7 (39)	1 (6)	8 (44)
12:00am II	225	40 (18)	0 (0)	13 (33)	0 (0)	27 (68)
Total	557	139 (25)	30 (22)	45 (32)	5 (4)	60 (43)

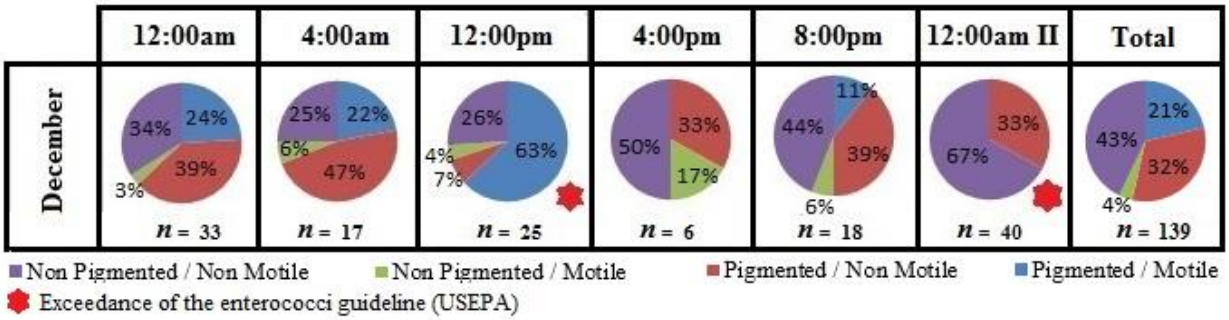


Figure 3.8. Frequencies of the pigmentation/motility groups in the enterococci community during December (2009).

On the other hand, the results for the month of March are summarized in Table 3.8. The frequency and distribution of the enterococci is presented in Figure 3.9. In this case, a total of 137 isolates, or 23% of the total enumerated community, were analyzed. The only exceedance event occurred at 8:00 pm and it was dominated by non pigmented-non motile enterococci. The sample collected before (4:00pm) and after (12:00am II) of the exceedance were also dominated by this group. Furthermore, like in December, the diversity in the samples changed at least every 4 hours.

Table 3.8. Pigmentation and motility groups of the analyzed enterococci in March (2010).

Sampling hours	Total Enumeration (CFU/100ml)	Analyzed isolates *n* (%)	Pigmentation and motility groups			
			Pigmented / motile *n* (%)	Pigmented / non motile *n* (%)	Non pigmented / motile *n* (%)	Non pigmented / non motile *n* (%)
12:00am	52	20 (38)	8 (40)	5 (25)	1 (5)	6 (30)
4:00am	48	17 (35)	4 (24)	8 (47)	1 (6)	3 (18)
8:00am	42	21 (50)	6 (29)	5 (24)	1 (5)	9 (43)
12:00pm	56	16 (29)	9 (56)	2 (13)	1 (6)	4 (25)
4:00pm	17	17 (100)	1 (6)	6 (35)	2 (12)	8 (47)
8:00pm	358	35 (10)	2 (6)	12 (34)	0 (0)	21 (60)
12:00am II	11	11 (100)	2 (18)	3 (27)	1 (9)	5 (45)
Total	583	137 (23)	32 (23)	41 (30)	7 (5)	56 (41)

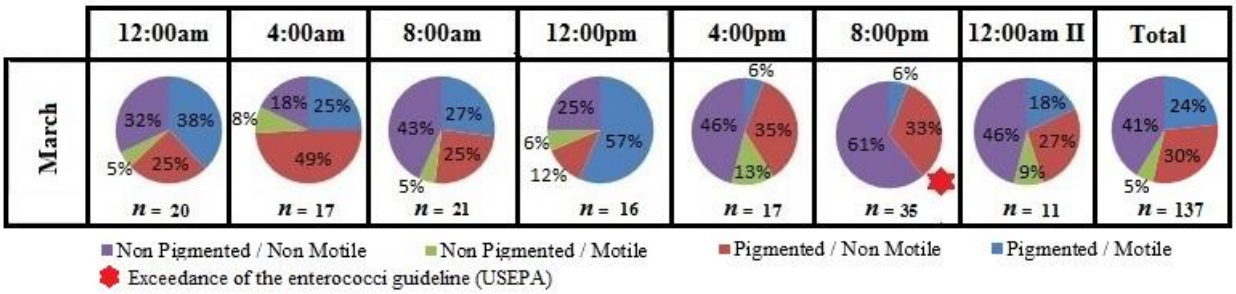


Figure 3.9. Frequencies of the Pigmentation / Motility groups in the enterococci community in March (2010).

In May, a total of 161, or 41%, enterococci isolates were analyzed (Table 3.9). The frequencies of the community distribution are summarized in Figure 3.10. The results showed that during this month the dominant enterococci were the non-pigmented, with 51% of the community. For this month we only observed one exceedance event at 4pm and 71% of the enterococci were non-pigmented non-motile. Interestingly, 4 hours later (8:00 pm) the total community was reduced by 73% and the dominant groups were the pigmented enterococci.

Table 3.9. Pigmentation and motility groups of the analyzed enterococci in May (2010).

Sampling hours	Total Enumeration (CFU/100ml)	Analyzed isolates *n* (%)	Pigmentation and motility groups			
			Pigmented / motile *n* (%)	Pigmented / non motile *n* (%)	Non pigmented / motile *n* (%)	Non pigmented / non motile *n* (%)
12:00am	64	13 (20)	8 (62)	0 (0)	0 (0)	5 (38)
8:00am	31	20 (65)	10 (50)	5 (25)	0 (0)	5 (25)
12:00pm	35	35 (100)	13 (37)	0 (0)	0 (0)	22 (63)
4:00pm	161	49 (30)	12 (24)	2 (4)	2 (4)	33 (67)
8:00pm	44	21 (48)	12 (57)	0 (0)	0 (0)	9 (43)
12:00am II	60	23 (38)	14 (61)	3 (13)	0 (0)	6 (26)
Total	395	161 (41)	69 (43)	10 (6)	2 (1)	80 (50)

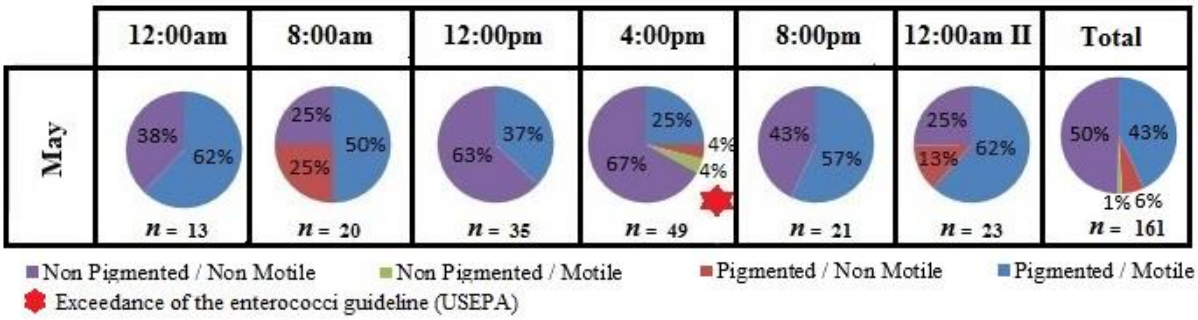


Figure 3.10. Frequencies of the Pigmentation / Motility groups in the enterococci community in May (2010).

In addition, the results of the study in June (Table 3.10) demonstrated that the enterococci community was dominated by the pigmented-motile group in 55% of the total 176 analyzed isolates. Even more, this was the dominant group during the two registered exceedance events, both at 12:00am. However, in contrast with the previous results, we did not observe the consistent variations of the enterococci community every 4 hours for this month. On the other hand, several catalase-positive bacteria were recovered in our samples that, although were discarded from the study, affected the percent values of the *n* analyzed at 12:00pm, 4:00pm and 8:00pm.

Table 3.10. Pigmentation and motility groups of the analyzed enterococci in June (2010).

Sampling hours	Total Enumeration (CFU/100ml)	Analyzed isolates *n* (%)	Pigmentation and motility groups			
			Pigmented / motile *n* (%)	Pigmented / non motile *n* (%)	Non pigmented / motile *n* (%)	Non pigmented / non motile *n* (%)
12:00am	144	24 (17)	11 (46)	5 (21)	0 (0)	8 (33)
4:00am	30	23 (77)	13 (57)	4 (17)	0 (0)	6 (26)
8:00am	71	3 (4)	2 (67)	1 (33)	0 (0)	0 (0)
12:00pm	3	5 (167)	2 (40)	0 (0)	0 (0)	3 (60)
4:00pm	12	23 (192)	17 (74)	2 (9)	0 (0)	4 (17)
8:00pm	3	7 (233)	2 (29)	3 (43)	1 (14)	1 (14)
12:00am II	469	91 (19)	49 (54)	19 (21)	0 (0)	23 (25)
Total	732	176 (24)	96 (55)	34 (19)	1 (1)	45 (26)

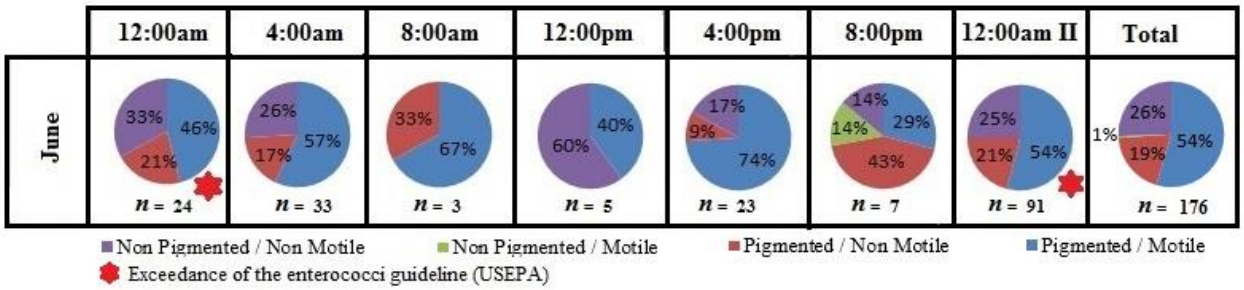


Figure 3.11. Frequencies of the Pigmentation / Motility groups in the enterococci community in June (2010).

Finally, in August, the results of the 209 analyzed isolates (Table 3.11) showed that the community was principally composed by two groups: (i) the pigmented-motile and (ii) the non pigmented-non motile. This also was supported by the overall results, in which the proportion of these two groups was nearly balanced (50% / 47%) whereas non pigmented-motile strains were poorly represented (3%). Interestingly, although these two groups also dominated in the two continuous exceedance events, they switched dominance at a 4-hour intervals. Specifically, from 65% non pigmented-non motile at 8:00pm to 56% pigmented-motile at 12:00am II.

Table 3.11. Pigmentation and motility groups of the analyzed enterococci in August (2012).

Sampling hours	Total Enumeration (CFU/100ml)	Analyzed isolates *n* (%)	Pigmentation and motility groups			
			Pigmented / motile *n* (%)	Pigmented / non motile *n* (%)	Non pigmented / motile *n* (%)	Non pigmented / non motile *n* (%)
12:00am	15	15 (100)	7 (47)	0 (0)	0 (0)	8 (53)
4:00am	41	27 (66)	8 (30)	0 (0)	1 (4)	18 (67)
8:00am	25	25 (100)	18 (72)	0 (0)	0 (0)	7 (28)
12:00pm	12	12 (100)	9 (75)	0 (0)	0 (0)	3 (25)
4:00pm	42	29 (69)	11 (38)	0 (0)	4 (14)	14 (48)
8:00pm	183	51 (28)	17 (33)	1 (2)	0 (0)	33 (65)
12:00am II	258	50 (19)	28 (56)	0 (0)	1 (2)	21 (42)
Total	576	209 (36)	98 (47)	1 (0)	6 (3)	104 (50)

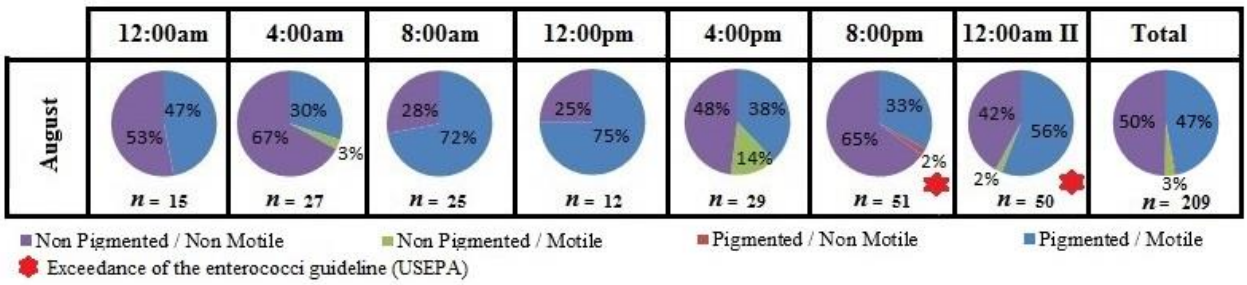


Figure 3.12. Frequencies of the Pigmentation / Motility groups in the enterococci community in August (2012).

3.3.5. Diurnal variation

In order to compare the enumeration and distribution of the enterococci community diurnal variation, we analyzed the results considering the sunlight as a possible influencing factor. The samples collected between 8:00am and 4:00pm were considered as corresponding to day time, while the night-time samples were those collected from 8:00pm to 4:00am.

Figure 3.13 shows that 76.3% of the enterococci were collected and enumerated at night. Furthermore, during the day the numbers of enterococci were lower and only represented 23.7% of the total enterococci enumerated during the project. Interestingly, all the four groups were collected more frequently during the night (Figure 3.14). The exceedance episodes were registered more frequently at night (75%) and none were at 8 am, the sampling time recommended by EPA. Moreover, 50% of the exceedance episodes were recorded at midnight.

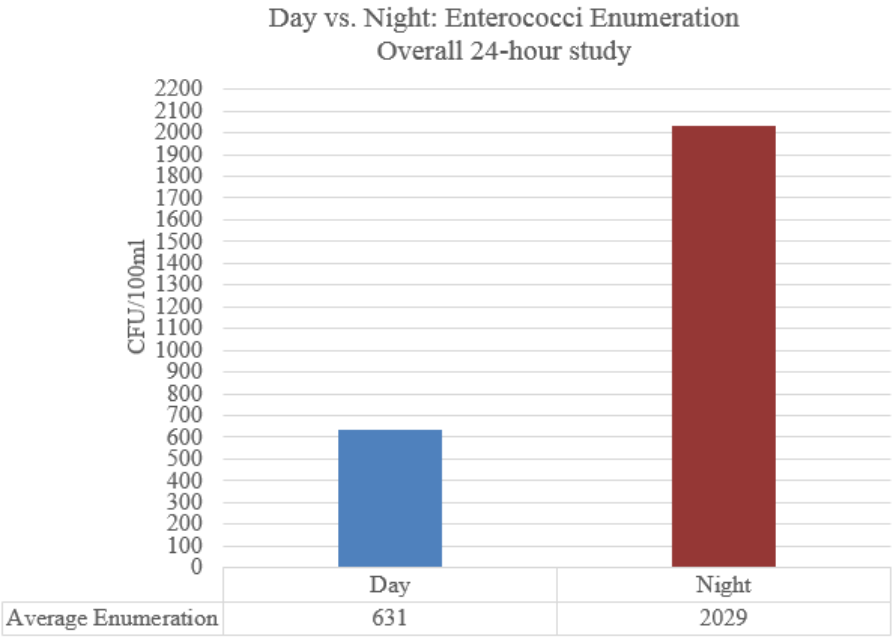


Figure 3.13 Comparison of the enterococci average enumeration (CFU/100ml) between day and night in the overall 24-hour study months.

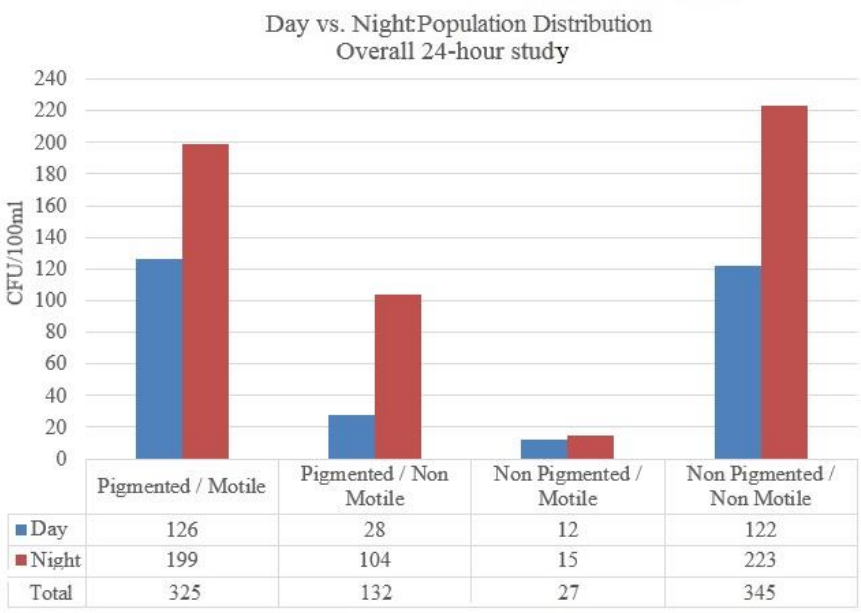


Figure 3.14 Distribution of the analyzed enterococci using Pigmentation / Motility groups between day and night in the overall 24-hour study months.

3.3.6. Before, during, and after the exceedance event

In view of the variability that was observed on the enterococci distribution through the 24-hour period at each month (section 3.3.1), a deeper study of the community was conducted. The main objective was to determine if the exceedance was caused by a recent introduction. As an alternative we assessed the enterococci species that were at before, during, and after the event (out of the permissible enterococci concentration levels) to determine if there were differences in the species distribution through these stages. The species identification was realized using the RFLP method (Chapter 2) and the enterococci isolates from the 24-hour study of May (2010) and August (2012) were used.

3.3.6.1. Distribution of the enterococci species

As previously reported in the average enumeration of May (Table 3.3), the enterococci concentration increased progressively from noon, reaching the highest value at 4:00pm

(exceedance event) and followed by a decrease in the community at 8:00pm. To attempt to understand these fluctuations in the culturable community through this exceedance event the enterococci were identified to the species level (Figure 3.15). In general terms, we observed that the community of enterococci was dominated primarily by two species: *E. casseliflavus* and *E. faecalis*. By following the enterococci community through time we observed differences in the distribution and dominance within before (A), during (B), and after (C) an exceedance event. First, the community had higher diversity of the species present at "during" than "before" and "after". Specifically, this high diversity was comprised by the dominance of *E. faecalis*, with half of the total community, and the occurrence of *E. casseliflavus* (24%), *E. faecium* (8%), *E. gallinarum* (4%) and two types of unknown species (14%) that were denominated as II (86% out of the 14%: 12.04%) and III (14% out of the 14%: 1.96%). The other two stages, "before" and "after", were dominated mainly by *E. faecalis* or *E. casseliflavus.*

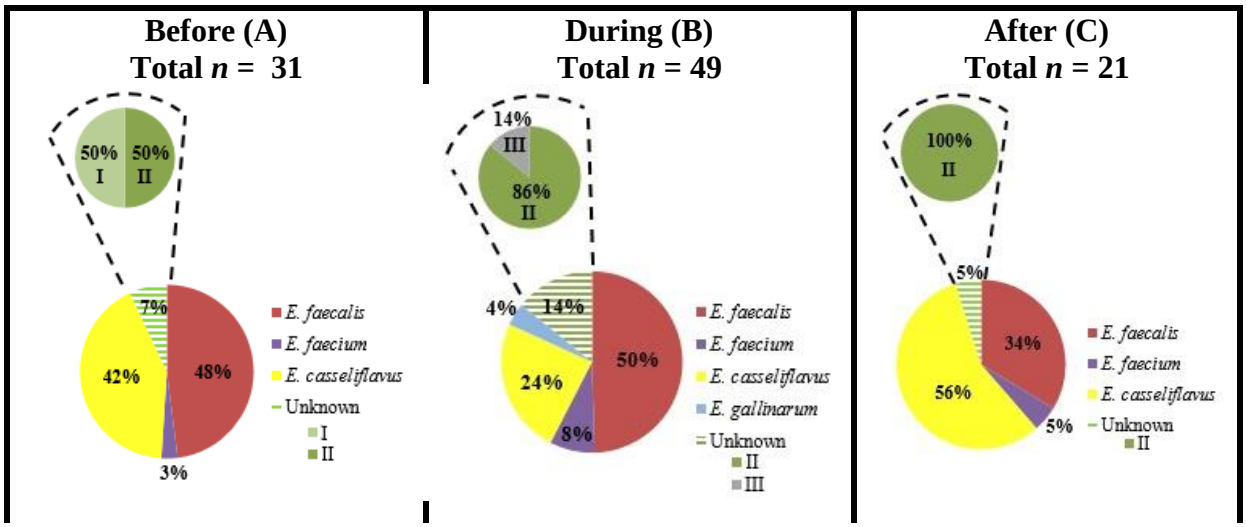


Figure 3.15. Species distribution of the enterococci community at before (A), during (B) and after (C) the exceedance event in May (2010).

The other exceedance event occurred in August (Table 3.5), but in that event we missed the "after" analysis since it happened at the end of our sampling schedule as seen in Figure 3.16.

The results show that the samples taken at "before" (A') and "during II" (C'), appear to be more diverse than the "during I" (B) sample. Furthermore, in both cases, the community was dominated by *E. casseliflavus*, followed by *E. faecalis*, two unknown species, and *E. faecium.* Interestingly, the two consecutive samples with concentrations of enterococci outside of the parameters showed a distinct community of enterococci. This exceedance event had a limited number of species that included *E. faecalis*, *E. casseliflavus*, and unknown VI with 53%, 44%, and 3% in order of abundance, respectively.

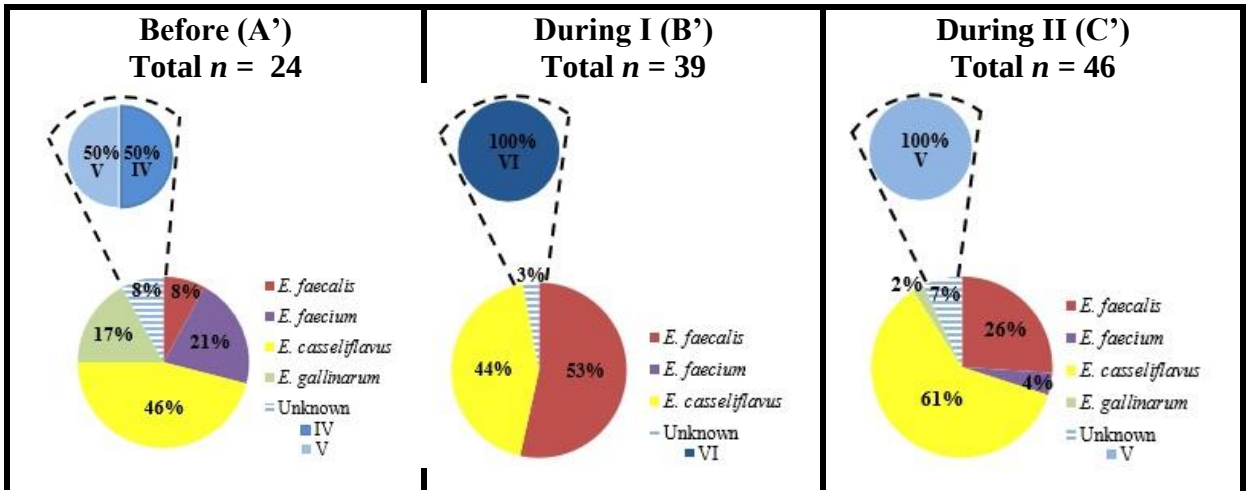


Figure 3.16. Species distribution of the enterococci community at before (A') and during I (B') and II (C') the exceedance event in August (2012).

3.3.6.2 Virulence Factors

Enterococci species identified at before, during, and after the exceedance event were analyzed for the presence of five virulence factors (*gelE*, *asa1*, *hyl*, *esp*, and *cylA*) to compare the genetic makeup of the same enterococci species that were identified through the event. However, considering that these genes are limited to *E. faecalis* and *E. faecium*, we concentrated the analysis only on these two species.

The expected amplification sizes of each virulence gen were: *asa1* at 375bp, *gelE* at 213bp, *cylA* at 688bp, *esp* at 510bp and *hyl* at 276bp. The Figure 3.17 shows an overview of the amplifications of the virulence factors genes mainly found in the environment including positive control *E. faecalis* MMH594 (lanes 1 and 15) used to confirm the results. However, considering that the positive control does not possess the *hyl* gene, and we did not have a positive control for this gene, we did not used this information since we could not determine if this gene was indeed present in the isolate or if the primer pair did not work.

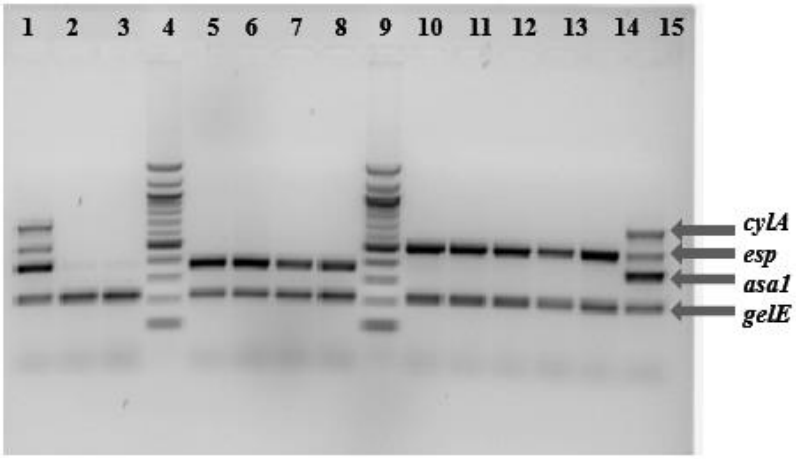


Figure 3.17. Enterococci virulence genes multiplex PCR: *gelE, asa1, esp, cylA.* Lane 1, Positive control *E. faecalis* MMH594; line 2, 5B; line 3, 7B; line 4, 100bp molecular marker (Promega); lane 5, 4D; lane 6, 9D; lane 7, 12D; lane 8, 13D; lane 9, 100bp molecular marker (Promega); lane 10, 5A; lane 11, 7A; lane 12, 9A; lane 13, 16A; lane 14, 17A; lane 15, Positive control *E. faecalis* MMH594.

The results from the samples collected in May (Figure 3.18) suggested that the *E. faecalis* community varied in content of virulence factors in the samples denominated as: "before" (A), "during" (B), and "after" (C) the event. For instance, the occurrence of the virulence genes found in the strains "before" were more variable than those from the other two time-points. Although the appearance of the combinations of *gelE/asa1* and *gelE/esp* were consistent through the three stages, *gelE* only and *gelE/asa1/cylA* were not. Regardless of the consistency reported for *gelE /*

asa1 and *gelE/esp*, it is important to notice that their frequencies differed throughout the event. Specifically, the genes combination of *gelE/asa1* dominated in the *E. faecalis* strains of "before" (71%) and "during" (75%), but not "after" in which, instead, the *gelE/esp* dominated (57%).

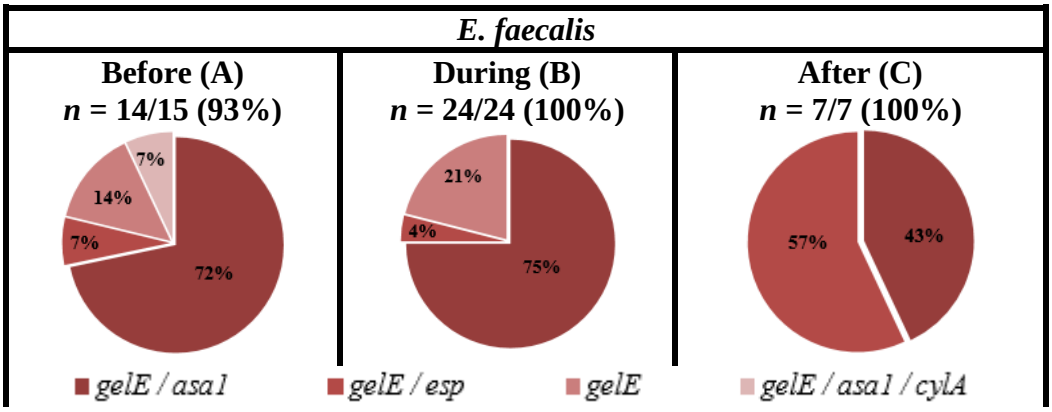


Figure 3.18 Virulence genes amplification of the *E. faecalis* strains from before (A), during (B) and after (C) the exceedance event in May (2010).

On the other hand, *E. faecium* (Figure 3.19) did not contain many virulent genes; their community was limited to *gelE* and *gelE/asa1*. In summary, even though we had limited numbers of analyzed isolates (*n*), the highest diversity was observed at "during" event, with the occurrence of *gelE/asa1* (50%) and *gelE* (50%).

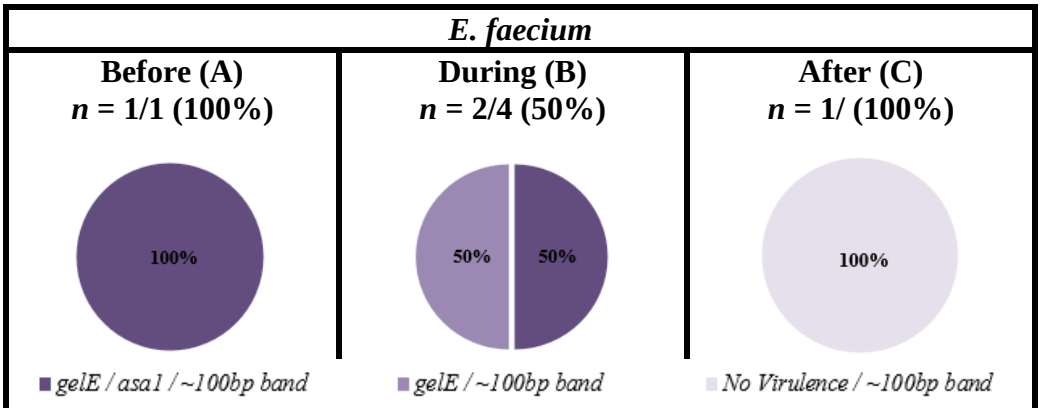


Figure 3.19. Virulence genes amplification of the *E. faecium* strains from before (A), during (B) and after (C) the exceedance event in May (2010).

The same analysis was conducted with non pigmented-non motile species from the August study. However, as previously explained, for this month we had a second "during" (demarked as II) instead of an "after" event.

First, considering the results of the *E. faecalis* strains (Figure 3.20), the highest diversity of virulence factors was observed at "during I" (B') in comparison with the other two stages. Specifically, the presence of *gelE* and *gelE/asa1* was consistent through the event, while *gelE/asa1/cylA* and *asa1/esp/cylA* were encountered only at "during I", although it was in a low frequency (5%). Basically, the amplification of *gelE* dominated in the analyzed community when the enterococci concentration exceeded the standard (B' and C').

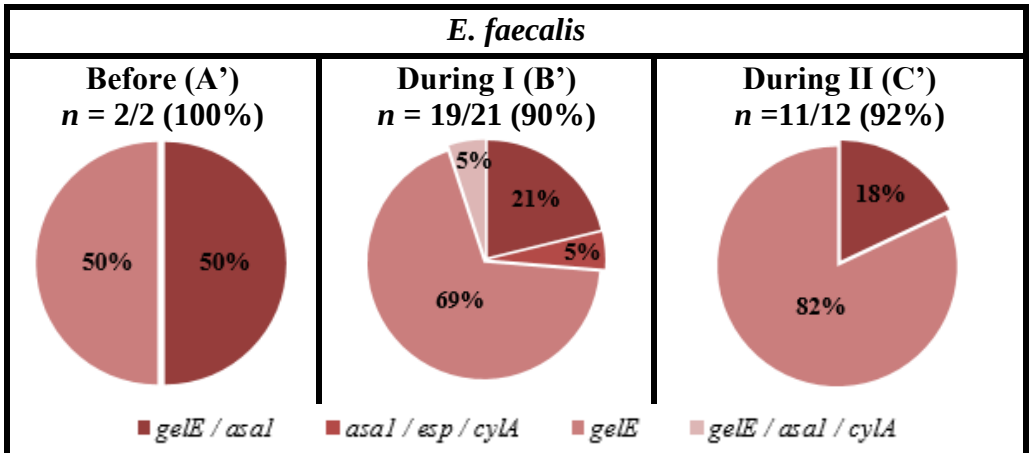


Figure 3.20. Virulence genes amplification of the *E. faecalis* strains from before (A') and during I (B') and II (C') the exceedance event in August (2012).

However, the presence of *E. faecium* (Figure 3.21) was limited to "before" and "during II" (A' and C'), considering that no *E. faecium* isolates were recovered at "during I" (B'). However, in spite of this limitation and the low analyzed strains, the diversity observed at "before" was higher (50% *gel/asa1* and 50% no virulence) than at "during II" (100% no virulence). Interestingly, as with the *E. faecium* analyzed from May samples (Figure 3.19), we also observed the unknown 100-bp fragment amplification in the previous analyzed strains.

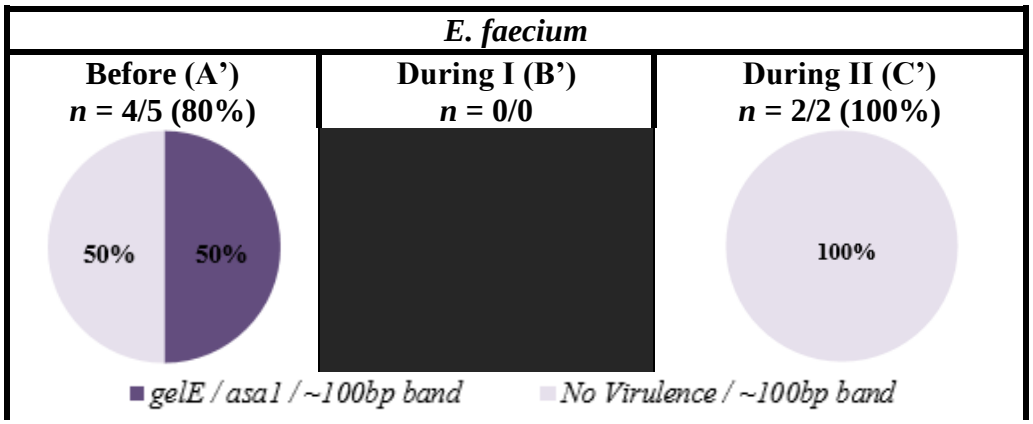


Figure 3.21. Virulence genes amplification of the *E. faecium* strains from before (A') and during I (B') and II (C') the exceedance event in August (2012).

3.4 Discussion and conclusions

Following the considerations issued by the USEPA for monitoring the recreational waters, they suggest that the sample collection must be done in the morning to best protect human health. That is because, according to their culture results, the highest indicator densities are observed at 8:00am, while, the lowest densities typically occur between 2:00pm and 3:00pm (USEPA, 2010).

In contrast, we observed in our 24-hour study that high enterococci enumerations were mainly obtained at night. Specifically, a total of eight exceedance events were observed and quantified, six of them took place at night (four at 12:00am and two at 8:00pm), while only two occurred during the day at 12:00pm and 4:00pm, respectively. In this context, we have to question not only the EPA's sampling time suggestion but also its accuracy to predict the cleanliness of the beach. In this study, we quantified the enterococci community in water samples from a beach every 4 hours for 24 hours, once a month, during five months. The total number of single samples analyzed was 105 and from these 15 corresponded to the 8:00am sampling time. None of the 15 samples were found to exceed the limit. In our case, adopting the EPA's recommendation would

have deemed this beach as clean and safe to use by bathers. We understand the limitations and the responsibility that the regulatory agencies endure to safeguard the public but our findings suggest that this method does not accurately predicts a recent contamination event. Furthermore, when we consider the total 21 samples collected each day of the study months and apply the latest recommendations by USEPA water quality criteria for the geometric means (USEPA, 2012), we concluded the opposite; the beach under study was not safe for bathers. Actually, this last conclusion holds true for every month we analyzed, but considering the numerical differences, the diversity and distribution of the enterococci community at 4 hours intervals where at 4:00pm the community was represented by 16 individuals (well within the limit), 4 hours later the samples exceeded the limit with 258 individuals, and 4 hours later, again, the number is back within limits with 11 individuals"), it is difficult to grasp a clear understanding of the origin of these organisms and if they really could be used to accurately predict possible harm to humans.

On the other hand, the standard deviation values demonstrated variations within the enumeration results recovered in the same sampling hour, even though the short collection time of the triplicates (less than two minutes). Specifically in December, we observed that some of the standard deviation ranges overlapped the USEPA guideline in the sense that one of the samples exceeded the standard even though the average enumeration did not. This demonstrated that the system composition could affect the sample enumeration or even worst, the water quality assessment. Furthermore, considering the patterns that were frequently found such: (i) high standard deviation while high enumerations and (ii) low standard deviation while low enumerations, we understand that the water system could move through a homogenous to

heterogeneous one in terms of the community composition by the impact of external factors unknown to us at this time.

Previous studies suggested that the consistent temporal and spatial variability of the enterococci community in recreational waters is influenced by external factors such as solar radiation, tides, and rainfall (Boehm, 2007; Enns et al., 2012). It has been proposed that high tides are responsible for increasing enterococci densities by resuspending bacteria attached to sand particles (Shibata et al., 2004; Desmarais et al., 2002); however, we were unable to observe this correlation in our data set. Our results demonstrated that the enterococci density varied constantly and that the tide levels did not seem to influence our results. In fact, we could observe high densities at low tides and low densities at high tides; more often than none, these did not explain the exceedance events observed.

Regardless of these fluctuations in community sizes, it should be noted that the enterococci concentrations, in general, were higher at night than during the day. For instance, overall, we found almost three times more enterococci at nighttime than during the daytime. Interestingly, other scientists have found these same results even in temperate waters (Boehm, 2007; Enns et al., 2012), but this observation is not enough to conclude that the solar radiation is solemnly responsible for the diminished enterococci community (Boehm, 2007; Enns et al., 2012; Sinton et al., 2002). However, in contrast with other studies that established the photoinactivation as a determinant factor in the species composition considering the pigmented phenotype as a competitive advantage (Maraccini et al., 2012), we observed no such thing among the enterococci community recovered in our study. In fact, all of the groups based on pigmentation and motility were isolated in higher numbers during the night. Furthermore, the overall dominant species among the pigmented

enterococci was *E. casseliflavus*, regardless of the variability in the enterococci concentrations or the solar radiation. This suggests that the yellow pigmentation is not necessarily an advantage during the sunlight exposure, but it seems that this organism is well equipped to survive in this environment. Likewise, *E. faecalis* was the most common non-pigmented enterococci in this environment, suggesting that among all other species in this group it is the best equipped to survive in the surveyed marine environment.

It is important to realize that *E. casseliflavus* is not typically the dominant *Enterococcus* species in human fecal matter; in fact this environment is dominated, by at least 3 to 4 orders of magnitude, by *E. faecalis* and *E. faecium* (Aarestrup et al., 2002; Tannock & Cook, 2002; Kühn et al., 2003). Our results demonstrated that although *E. faecalis* and *E. faecium* were present when the enterococci densities exceeded the USEPA guidelines in our study site, they were not the dominant community in all of the "recent contamination events", and, actually, they shared the dominance with *E. casseliflavus* (4 out of 8 exceedance events). Interestingly, when *E. casseliflavus* dominated during the exceedance event, 3 of these 4 events were at midnight. Likewise, the non-pigmented non-motile enterococci group, dominated by *E. faecalis*, was responsible for the remaining exceedance events and 3 of the events were at night also. Even more, the enterococci community was extremely dynamic acquiring and losing species diversity before, during, and after an exceedance event. However, although our findings showed that this diversity is limited to *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus* and other unknown species, these were, except *E. casseliflavus*, also the predominant species reported in sewage (Manero et al., 2002).

Since these enterococci species are predominant in sewage, a high diversity of genes should be expected in a fecal contamination event, considering the mixture of different strains and sources. This was not observed in our results, in which the presence of four virulence genes in *E. faecalis* and *E. faecium* had no significant variation when the enterococci concentrations exceeded or not the USEPA standard. This does not mean that the exceedance did not represent a fecal introduction; this means that neither the species nor the strains were different during the event, and before or even after it.

Finally, we observed that the enterococci community was highly dynamic, in concentration and distribution, through a 24-hour period. Even though these communities of enterococci varied in numbers and group composition in a relatively short period of time, this environment was limited to four main species regardless of whether the enumeration exceeded or not the EPA guidelines. In addition, although these limited species were mainly dominated by *E. casseliflavus*, the human-related species *E. faecalis* and *E. faecium* were always present. However, interestingly too, none of these two "warning" species presented significant differences in their virulent factors as we expected to find during a "recent fecal contamination" event.

In conclusion, if we consider only the USEPA regulations, single or geometric mean, we should determine that this beach seems to be contaminated and pose a health risk to bathers. On the other hand, if we consider our overall results we might suggest that those exceedance events were not necessarily due to a recent fecal contamination event. Although further studies are needed to determine the source of introduction, fecal or not, we have to conclude that a single numerical

value is not a reliable method to identify the potential of health risks by fecal contamination in a subtropical recreational water system.

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