ISOLATION AND IDENTIFICATION OF THE FECAL POLLUTION INDICATORS *Enterococcus* spp. FROM SEAGRASS *Thalassia testudinum* AT LA PARGUERA PUERTO RICO and THE USE OF ENTEROCOCCI FOR MICROBIAL SOURCE TRACKING

By

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ABSTRACT

The occurrence and persistence of the fecal contamination indicators *Enterococcus* spp. were studied in La Parguera, southwestern Puerto Rico, as an initial step in microbial source tracking (MST). The scientific literature suggests that different enterococcal species are associated to specific homeothermic hosts. Results from this study indicate birds as the main sources of fecal pollution in the area and suggests a *E. gallinarum* - cattle egret association. Precipitation may be a major factor in enterococcal occurrence. There are also reports that indicate that enterococci might survive for extended periods of time in sediments and in the phylosphere of vegetation. Potential non-point sources of enterococci may cause an overestimation of the enterococci that suggest fecal pollution as well as an obstacle for MST. In consequence, the association and survival of enterococci in the seagrass *Thalassia testudinum*, was evaluated. It was found that *T. testudinum* is able to host enterococci. However, overtime persistence of enterococci in this ecosystem was not evident. During the development of this study, high numbers of false positive enterococci were found while using standard methods for the isolation of enterococci from the environment. A protocol using Enterococcosel Broth followed by two confirmation tests was examined as a potential approach to be used in monitoring coastal waters of Puerto Rico. The method presented is economically viable, but still needs improvement. A re-evaluation of the current methods for fecal contamination assessment in our coastline is suggested.

RESUMEN

La presencia y persistencia de los indicadores de contaminación fecal, *Enterococcus* spp. fueron estudiados en la Parguera, al suroeste de Puerto Rico como un paso inicial a la técnica de rastreo de fuente microbiana (MST, por sus siglas en inglés). La literatura científica sugiere que algunas especies de enterococos están asociadas a hospederos homeotérmicos específicos. Los resultados de este estudio indican a las aves como las fuentes principales de contaminación fecal en el área y sugieren además una correlación entre *E. gallinarum* y las garzas. La precipitación también presentó ser un factor primordial en la presencia de enterococos. Existen reportes que indican que algunas de las especies de enterococos pueden sobrevivir en asociación a sedimentos y a la vegetación por periodos extensos de tiempo. Fuentes alternas de enterococos pueden ocasionar un sobre-estimado de los enterococos que indican contaminación, al igual que un obstáculo para MST. En consecuencia, la asociación y sobrevivencia de los enterococos en la yerba marina *Thalassia testudinum*, fueron evaluadas. Se encontró que *T. testudinum* puede ser un hospedero de enterococos. Sin embargo, la persistencia de estos organismos a través del tiempo en este ecosistema no fue evidente. Durante el desarrollo de esta investigación, se documentó un número alto de falsos positivos al utilizar metodologías sugeridas para aislar enterococos del ambiente. Se examinó un protocolo utilizando el caldo nutritivo Enterococcosel, seguido por dos pruebas de confirmación como un método potencial para ser utilizado en el monitoreo de las aguas costeras de Puerto Rico. Aunque el método demostró ser económicamente accesible, aún necesita ser mejorado. Se sugiere una re-evaluación

de los métodos actuales que son utilizados para evaluar la contaminación fecal en nuestras costas.

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CHAPTER 1: Introduction

1.1 Introduction

Fecal contamination impairs the health of coastal ecosystems by modifying carbon and nutrient cycling, and minimizing their potential for safe recreational water dependent activities. In addition, microorganisms related to fecal contamination may cause human diseases such as meningitis, hepatitis A, gastrointestinal illness, among others (Desmarais *et al*., 2002; Kinzelman *et al*., 2003; Scott *et al*., 2002). Increasing human population density together with unsustainable economic activities are some of the principal causes of many forms of contamination. Major sources of fecal contamination in communities established in areas with shallow ground water levels include overflowing from sewer systems, sewage treatment plants, and septic tanks. Other compounding factors include poorly designed urban development and animal farming close to creeks and rivers. Overall, fecal contamination in coastal systems degrades environmental quality thus affecting natural nutrient cycle, wildlife, and tourism.

The use of fecal pollution (FP) indicators is important in preventing and protecting coastal communities from microbiological outbreaks. A good indicator microorganism must be easily isolated, be related to warm-blooded animals digestive system, and represent an actual FP event (Scott *et al*., 2002; Shanks, 2005). The U.S. Environmental Protection Agency (U.S. EPA) suggests the use of fecal coliforms, *Escherichia coli*, and the genus *Enterococcus* as FP markers (Griffin, 2001). These organisms are normally found in the intestines of warm-blooded animals. It is suggested that enterococci in seawater are better indicator of FP because of their ability to survive

in stress conditions such as salinity, temperature*, and* pH variations (Bordalo *et al.*, 2002; Stewart *et al.* 2007).

In addition, a good FP indicator must not be able to replicate outside the intestines of warm-blooded animals (Scott *et al.*, 2002). Enterococcal persistence and capacity of replication have been well documented for sediments and some plants (Desmarais *et al.*, 2002; Hartel *et al.*, 2004; Müller *et al.*, 2001; Ott *et al.*, 2001; Whitman *et al.*, 2003, 2004). Some studies indicate that in temperate and sub-tropical coastal areas affected by a large tidal range, *E. coli* and enterococci can survive and even replicate on desiccated and rewetted sediments (Desmarais *et al.*, 2002; Hartel *et al.*, 2004). There are also reports that indicate that these bacteria are capable of surviving on the phyllosphere of freshwater and sun-dried algae (Müller *et al.*, 2001; Ott *et al.*, 2001; Whitman *et al.*, 2003, 2005). Survival of enterococci in the environment produces another potential source of fecal indicator organisms, therefore decreasing the prospective usefulness as unique indicators of human derived fecal contamination. Thus, the existence of potential natural reservoirs of enterococci indicates that during certain conditions, the presence of these microbes may not be a reliable point and non-point source indicators of fecal contamination (Hartel *et al.*, 2004; Whitman *et al.*, 2003).

Although enterococcal isolation from natural environments is of high concern for FP assessment, the different species of this genus can function as tracers of the source of contamination. There are more than 25 identified *Enterococcus* spp. from different hosts. Some enterococci have been associated with specific warm-blooded hosts (Baele *et al.*, 2002; De Graef *et al.*, 2003; De Vaux *et al.*, 1998; Naser *et al.*, 2005; Ŝvec

et al., 2005b; Wheeler *et al.*, 2002). For example, an *Enterococcus faecalis* - *E. faecium* -*E. hirae* combination can be indicative of human related FP (Bonilla *et al.*, 2006). Consequently, the enterococci might be appropriate for source tracking. Microbial Source Tracking (MST) is used for detecting the main sources of pollution by matching a FP indicator with a particular animal source such as human, farm animals, and wildlife, by applying microbiologic, genotypic, phenotypic, and/or chemical methods (Scott *et al.,* 2002, Harwood, 2007). Source tracking methods are essential when adopting decisions on the pertinent actions to solve a FP event, by pinpointing specific source location and type (Stewart *et al.,* 2007).

Based on the above, three main questions were addressed during this work: **(i)** Is, the seagrass *Thalassia testudinum* a potential host or reservoir of human related enterococci in tropical coastal waters?, **(ii)** Is the enterococci community found in the water column similar to those found associated to *T. testudinum*?, and **(iii)** What possible sources of fecal input are detected in the coastal region of La Parguera during the study period? Occurrence of a high number of false positive enterococci in many of our samples while using standard methodologies (Hartel *et al.*, 2005; USEPA, 2002) led to the fourth question: **(iv)** Are we using the right approaches for FP assessment in our tropical coastlines?

The current study was performed at La Parguera, a fishing village in Lajas, in the southwest coast of Puerto Rico. Its rich variety of marine ecosystems makes it an attractive place for fishermen and tourists (Boschetti-Aponte, 2002). Human-induced activities at La Parguera, together with natural environmental variability, have increased

the chance of FP events associated to human (i.e. manholes, treatment plant, and tourism) and non-human (i.e. wild and farm related birds, dogs, horses, monkeys, among others) sources.

1.2 Goals and Objectives

(i) Is, the seagrass *Thalassia testudinum* a potential host or source of human related enterococci in tropical coastal waters?

Previously described survival of fecal indicator organisms in natural environments (Desmarais *et al.*, 2002; Hartel *et al.*, 2004, 2005; Hartke *et al.*, 1998, Whitman *et al.*, 2003) encouraged the investigation of the role of the seagrass *Thalassia testudinum* as a potential underwater reservoir and source of enterococci. *Thalassia testudinum* is a dominant component of shallow coastal benthic habitats in the Caribbean (Detrés *et al.*, 2001; García-Ríos, 2001). The proximity to the shore of this seagrass species makes it susceptible to input of a variety of contaminants and in consequence, increases the opportunity of colonizing bacteria and epiphytes (Newell, 1981). The influence of fecal contamination on the microbial community composition associated to the phyllosphere of underwater vegetation is uncertain.

It was of interest to study *Thalassia testudinum* as a natural reservoir and as a potential source of *Enterococcus* spp., in order to evaluate enterococci viability as indicators of recent FP events. No known report on the capacity of underwater marine plants to constitute reservoirs of enterococci is available. Specific objectives related to this question were:

1. To understand enterococcal species diversity on *T. testudinum* over different time periods and environmental conditions.
2. To examine the possibility of *T. testudinum* as a host or as a source of human related enterococci.
3. To estimate the persistence of human-related *E. faecalis* when inoculated into *T. testudinum*.
4. To evaluate the environmental factors that might affect the capacity of attachment of human related enterococci when directly inoculated to *T. testudinum* beds.

To fulfill these objectives, and test the hypothesis of *T. testudinum* as an underwater reservoir of enterococci, seagrass and seawater samples were collected at different locations and dates. Also, *E. faecalis* ATCC 29212, a strain isolated from human urine, was directly inoculated on *T. testudinum* in two different assays, including inoculation on a natural environment and on fish tanks.

(ii) Is the enterococci community found in the water column similar to those found associated to *T. testudinum*?

It was of interest to evaluate differences between enterococcal populations in seawater and those potentially found in the phyllosphere of *T. testudinum* in order to evaluate the seagrasses as potential sources of non-pollution indicator enterococci. Non-human related enterococci have been isolated from different terrestrial vegetation sources (Martínez-Murcia and Collins, 1991; Müller *et al.*, 2001). Seagrasses are

present at sites frequently visited by human. Disturbance of these marine ecosystems by boating and trampling may provide a mean of dispersal of epiphytic bacteria.

The specific objectives related to this question were:

1. To corroborate if there is an enterococcal specie related to *T. testudinum* by identifying the variety of enterococcal species occurring at La Parguera.
2. To investigate the spatial and temporal dynamics of enterococci on seawater and *T. testudinum* in La Parguera.

(iii) What possible sources of fecal input are detected in the coastal region of La Parguera during the study period?

It was of interest in this work to study the occurrence and species composition of enterococci in order to identify possible human and wildlife sources of these microbes, as well as the environmental factors that might affect enterococcal abundance and/or diversity. It was expected, as suggested by previous publications, to find higher abundances of enterococci as we get closer to the shore as well as during precipitation events (Harwood, 2007; Lipp *et al.*, 2001). Seasonality in Puerto Rico is characterized by episodes of dryness and rain events (Detrés *et al.*, 2001) and by moderate daily temperature changes. The effect of these periodic changes on fecal bacterial diversity in tropical coasts is unclear. This was done in order to identify "hot spots" of feces input and to promote a MST approach as a tool to help control and prevent fecal contamination in Puerto Rico's marine coastal areas. This goal addressed the viability of

using enterococci as a FP indicator for MST in our coastal waters. It was of interest to match the isolated enterococci with specific environmental conditions, such as rain or dry periods, presence of bird rookeries, and/or anthropogenic inputs, that were evident during the study period. The specific objectives were:

1. To corroborate the use of enterococci as a source tracker of human and non-human related FP by pairing specific species to known environmental factors or patterns.
2. To establish a baseline of the diversity of enterococci present during the period of the study as a preamble of a library dependent Microbial Source Tracking (see below) approach for the study area.

(iv) Are we using the right approaches for FP assessment in our tropical coastlines?

Comparison with simultaneous water and sediment sampling conducted in coordination with the University of Georgia and the University of New Hampshire showed high number of false positives in water and sediment isolates while using enterolert (IDEXX), one of the suggested enterococcal isolation methods (Hartel *et al.*, 2005). During that investigation, a membrane filtration method with m-Enterococcus agar (Levin *et al.*, 1975) was also used, frequently resulting in filter clogging and overgrowth of non-targeted bacteria. These methodological difficulties suggested that the use and applicability of standard accepted methodologies should be re-examined. Therefore, a protocol using Enterococcosel Broth (EB) followed by confirmation tests with BHIA- 6.5%NaCl and catalase test, was examined as a potential approach to be used coastal waters of Puerto Rico. Specific objectives related to this question were:

1. To evaluate a most probable number (MPN) approach with EB as a possible inexpensive tool for the isolation of enterococci from marine environments.
2. To estimate the necessary confirmation tests after applying the MPN-EB method by considering the occurrence of false positives.

1.3 Literature Review

1.3.1 Microbial Source Tracking

Fecal pollution (FP) is a serious environmental concern particularly around coastlines used for recreation and economic purposes (Desmarais *et al.*, 2002, Griffin, 2001). Use of FP indicator microorganisms has been essential in identifying human and environment health risk areas. Microbial fecal indicators are bacteria from warm-blooded animal intestines and must represent fecal pollution events (Shanks, 2005). Feces-related microorganisms such as enteric bacteria and enterococci are traditionally used to indicate FP, but not its specific source.

Microbial source tracking (MST) is used as a tool for predicting and identifying the main hosts of fecal contamination, thereby facilitating decisions related to which approaches will be applied for an effective control of contamination (Stewart *et al.*, 2007). The concept of MST refers to match a FP indicator microbe from a polluted site with an animal source to suggest the origin of contamination; either if is human induced (ie. from farming, human, etc.) or if it is a wildlife contribution (Kuntz *et al.*, 2004; Shanks, 2005; Wheeler *et al.*, 2002). The main assumption for MST is that intestinal microbes of the variety of animal groups are expected to be different because of nutrient

selection (ie. space, available nutrients) and because of gut conditions (ie. temperature, diet, digestive system) (Shanks, 2005). An example on the use of MST is presented by Shanks (2005). It was estimated that in the year 2005, the USA produced around 1 x 10^{12} kg/year of feces. This production was dominated by beef cattle with a 44%, followed by chickens, pigs, and dairy with a 22%, 20%, and 10% respectively. The human contribution was of 0.7%. Other animals such as calves, sheep, turkey, dogs, and cats accounted for a 3.3% in total (Shanks, 2005).

Methods for MST are a supplement of sanitary surveys of beach contaminants and human health risks from the analysis of human versus non-human and human versus domestic animal sources (Shanks, 2005; Stewart *et al.*, 2007). The idea of discriminating between human and animal source of contamination dates from the 1960's, but the term MST has been recently used to define the variety of methods used for the identification of the origin of the contamination (Santo Domingo *et al.*, 2007). Techniques for MST have been developed because of the necessity of controlling the input of organic matter as well as pathogenic microorganisms into recreational waters (Scott *et al.*, 2002; Wheeler *et al.*, 2002). Techniques defined under MST include microbiological, genotypic, phenotypic, and chemical methods, each one presenting its own advantages, disadvantages, and improvement challenges. Scott *et al.*, (2002) present the use of fecal bacteria as well as enteric viruses, phages, and chemical compounds like sterols, stanols, and caffeine as indicators of human derived FP. The use of optical brighteners from laundry detergents has been also used as a first step in

the identification of potential sources of FP (Hartel *et al.*, 2007). However, only microbiological techniques will be discussed in this section.

Ideal source identifiers are microbial populations that are particular to a specific animal host, abundant in that host, and present temporal stability and geographic continuity (Shanks, 2005). Scott *et al.*, 2002, also suggests that a good microbial source indicator must be normally found in healthy individuals, rapidly detected and enumerated, and be unable to replicate outside of the intestines of warm-blooded animals. It might be non-pathogenic to humans, strongly associated with the presence of pathogenic microorganisms, and have similar surviving characteristics to those pathogens (Griffin, 2001). Although these specifications are necessary for a better understanding of environmental fecal pollution hazards, there is no source identifier that presents all the requirements of an ideal indicator (Griffin, 2001; Harwood, 2007; Scott *et al.*, 2002).

Microbes that are typically used as FP indicators include *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringes*, and total fecal coliforms, as well as some viruses (Griffin, 2001). Microbiological techniques used for MST are classified under library-dependent (LD) vs. library-independent (LI) methods which can also be defined under a variety of qualitative vs. quantitative descriptions and phenotypic vs. genotypic analysis (Shanks, 2005). Library dependent methods are databases of culture dependent isolates of *E. coli* or fecal enterococci. Examples of LD techniques include antibiotic resistance analysis, carbon utilization profiles, RFLP, and repetitive-PCR

(Harwood, 2007). These methods require thousands of isolates from water and suspected animal sources, and the pollution source identification can last 1-5 days. They are typically easy to perform and interpret and some are relatively inexpensive and reproducible. Disadvantages of LD include culture dependability and geographical and temporal specificity (Shanks, 2005).

Library independent methods do not require a database and requires of genotypic characterization (Harwood, 2007). Microbes isolation is not required in many of the LI techniques and source identification in this case can last from 6-8 hours (host specific PCR) to 1 month (total community analysis). Other microorganisms different from *E. coli* and enterococci are also considered when performing LI approaches. Although current LI methods can be rapid and sensitive, they present a variety of disadvantages such as high costs of performance and protocol challenges when defining host/microbe interactions (Shanks, 2005). Both, LD and LI approaches are still under development. Research still continues in order to develop better water quality assessments by identifying FP main sources. Microbial Source Tracking has shown to be promising and necessary for maintaining a healthy environment.

1.3.2 The Enterococci

Enterococcus spp. have been successfully used as a precise indicator of health risk in marine and recreational waters (Griffin, 2001). Enterococcal species are also suggested to be used for MST due to their host specificity (Baele *et al.*, 2002; De Graef

et al., 2003; De Vaux *et al*., 1998; Naser *et al*., 2005; Svec *et al*., 2005a, 2005b, 2006; Wheeler *et al*., 2002).

The Gram-positive species that belong to the *Enterococcus* spp. are facultative anaerobic bacteria commonly found as part of the intestinal flora of warm-blooded animals. Enterococci were originally classified as *Streptococcus* species until Schleifer and Kilpper redefined them as the genus *Enterococcus* in 1984 (Law-Brown and Meyers 2003, Müller *et al.,* 2001). They are still classified as group D streptococci, which means this group possess a specific cell wall carbohydrate (glycerol teichoic acid linked to cytoplasmic membrane). They are usually presented as short chains of rounded cells. They differ to other streptococci in that they can tolerate 40% bile, a pH of 9.6, and 6.5% NaCl (Cai, 1999), but recently described species present slow or non-growth at this salt concentration (Ŝvec *et al*., 2006). *Enterococcus* spp. survive at a temperature range of 10-45°C, being 37°C their optimal growing temperature, but *E. faecium* can survive at up to 60°C for ≥30 minutes (Holt *et al*., 2000; Tankson *et al*., 2002). They do not have the ability of forming spores and are mainly catalase negative (Carvalho *et al*., 1998; Facklam *et al*., 1989; Holt *et al*., 2000; Manero and Blanch, 1999), with rare exceptions (Müller *et al*., 2001; Ŝvec *et al*., 2001). *Enterococcus* species are also classified into five different groups (Appendix 7.1) according to their ability of using arginine and fermenting sorbose and mannitol (Facklam *et al*., 2002).

Enterococci are used worldwide as FP indicators to monitor recreational waters (Griffin, 2001) because they are relatively rapidly detected and their survival in water bodies is more related to known fecal pathogens when compared to *Escherichia coli*

(Kinzelman *et al.,* 2003). According to the U.S. EPA and the Puerto Rico Environmental Quality Board (PREQB), marine waters used for recreation (i.e. beaches) should not have more than 35 colonies of enterococci in 100 mL of sample (JCA, 2003). These counts are based on a geometric mean of five sequentially taken representative samples within a month (Griffin, 2001, Kinzelman *et al.*, 2003). For a single day sampling, the accepted enterococci limit for marine environments is 104 CFU/100 mL (Griffin, 2001).

Some enterococci have demonstrated some selectiveness in certain warm-blooded animal (Appendix 7.1), what makes them suitable for MST. The specie *E. columbae*, for example, is basically exclusive to pigeons (Baele *et al.*, 2002; Devriese *et al.*, 1990), while *E. canintestini*, *E. phoeniculicola*, and *E. asini* have been isolated from dogs, wood hoopoes, and donkeys respectively (DeVaux *et al.*, 1998; Law-Brown and Meyers, 2003; Naser *et al.*, 2005). The range of enterococci is different among different hosts, a typical characteristic that can be useful in MST (Figure 1.1). *Enterococcus faecalis* is suggested as indicator of human derived FP, although it can occur in a variety of hosts (Kuntz *et al.*, 2004; Wheeler *et al.*, 2002).

Microflora associated to the intestines changes during lifetime. This is also true for enterococci in the different stages of animals' development. *Enterococcus faecalis* have showed to be in equal proportions to *E. faecium* during the first days of life of chickens from industrial farms. This ratio varies during chicken development, being dominated by *E. faecium* during the first month of life and by *E. cecorum* over the 3 months of age (Devriese *et al.*, 1991). Kuntz *et al.*, (2004), suggest that when sampling

fecal polluted areas, if *E. faecalis* is the dominant enterococcal specie, it should be indicative of human derived FP, because its range in wild and farm birds is variable. Bonilla *et al.*, (2006), suggest that in a FP event when *E. faecalis* occurs as the most common specie followed by *E. faecium* and *E. hirae*, this correlation can be indicative of human related FP for sub-tropical areas.

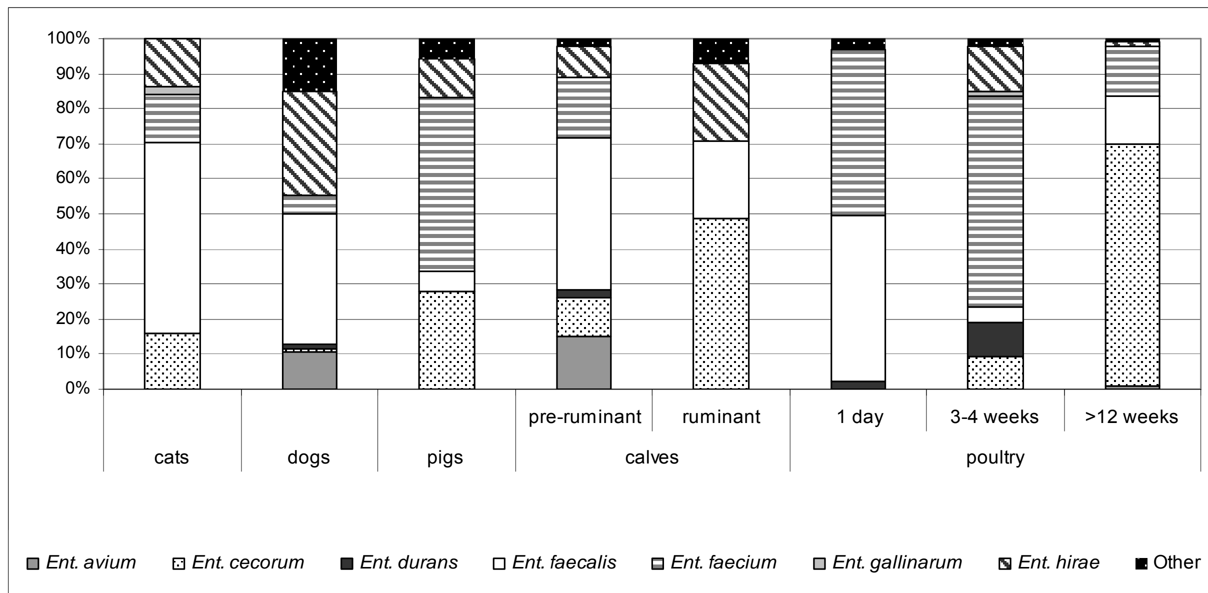


Figure 1.1. Distribution of different enterococci among selected farm and domestic animal species. Enterococci from calves and poultry are described for different stages of their development. The enterococci from cats, dogs and pigs are isolates from the anus and feces (Adapted from Aarestrup *et al.*, 2002 and Devriese *et al.*, 1991).

Although *Enterococcus spp*. are mainly associated to the digestive system of warm-blooded animals, they can also occur in other environments. Enterococci are commonly isolated from food (Grazia Fortina *et al.*, 2004), invertebrates (Cox and Gilmore, 2007; Silva *et al.*, 2003), vegetation (Müller *et al.*, 2001; Whitman *et al.*, 2005), water (Ŝvec *et al.*, 2005), and soils (Desmarais *et al.*, 2002; Hartel *et al.*, 2004, 2005).

1.3.3 Enterococcal occurrence and persistence in the environment

Studies on the occurrence of enterococci in the environment date from the 1940's. By that time, plant-specific variants of enterococci were suggested (Mundt, 1963). Since then, different enterococci have been isolated from leaves, flowers, and roots of a variety of plants, including those enterococci related to human and other warm-blooded animals (Jha *et al.*, 2005). The most common and abundant enterococcal species found on vegetation include *E. faecalis*, *E. casseliflavus*, *E. hirae*, *E. mundtii*, *E. faecium*, and *E. sulfureus* (Aarestrup *et al.*, 2002; Cai, 1999; Müller *et al.*, 2001; Ott *et al.*, 2001). Some of these species are prevalent in a variety of plants and present a yellow pigmentation when inoculated on laboratory media, a characteristic that has been suggested for identifying non-human sources of enterococci (Aarestrup *et al.*, 2002; Bahirathan *et al.*, 1998). Other enterococci are mainly associated to warm-blooded animals indicating that fecal pollution promotes direct and indirect bacterial phyllosphere colonization. Whitman *et al.*, 2005, suggest that the presence of enterococcal cells in the pitcher plant *Sarracenia purpurea* is due to insects. Some insects such as fruit flies might be carriers of enterococci when associated to areas that are susceptible to input of feces (Cox and Gilmore, 2007).

Certain enterococcal species, not associated to a direct fecal input, have been isolated from water. *E. moraviensis* and *E. haemoperodixus* were first discovered on fresh water sources in the Czech Republic (Svec *et al.*, 2001), while *Enterococcus*

aquimarinus was described from seawater samples from Italy in 1984 (Svec *et al.*, 2005a). These three species have not been previously described as components of warm-blooded animals. In marine environments, enterococci have also been isolated from plankton and oysters. *Enterococcus faecalis* can attach to chitin-containing zooplankton as a survival strategy when released into seawater. These were detected at the Adriatic Sea, only by quantitative-PCR and showed to be season-dependent, being more common during summer time (Signoretto *et al.*, 2004). Enterococci have been also isolated from oysters, where *E. faecalis, E. sulfurous, E. durans*, and *E. hirae were* 68, 3, 3, and 1 percent of the total number of isolates (Silva *et al.*, 2003).

One assumption when using a fecal indicator microorganism is that they are not able to persist for extended periods once released from the digestive system of warm-blooded animals (Anderson *et al.*, 2006). The long term persistence of enterococci under conditions of sediment and vegetation desiccation has been described (Desmarais *et al.*, 2002; Hartel *et al.*, 2004; Whitman *et al.*, 2003). On temperate and sub-tropical zones, *Enterococcus spp*. have persisted and replicated after rewetting dried clayey sediments (Hartel *et al.*, 2005). Whitman *et al.* (2003), also showed enterococcal recovery after rewetting samples of *Cladophora glomerata*, a green alga associated to freshwater environments, posterior to a period of 6 month of desiccation. Microbial attachment and diversity on the phyllosphere is influenced by the environmental conditions surrounding the leaves, which might include seasonality and exposure to contamination (Lindow and Brandl, 2003). Non natural environments can also be capable of sustaining enterococci. *Enterococcus faecalis* and *E. faecium* strains

survived for >90 days in cotton and polyester fabrics, suggesting the ability of these human-related species to persist in natural and synthetic environments (Neely and Maley, 2000).

There are a variety of mechanisms that help explain enterococcal survival in the environment. Enterococci tolerance to adverse environmental conditions (Hartke *et al.*, 1998; Signoretto *et al.*, 2000) and the spreading from fecal sources by the action of insects (Whitman *et al.*, 2005) and contaminated sediment wash off (Desmarais *et al.*, 2002), have been described as influencing factors of enterococcal dispersion. Occurrence on different animal sources such as insects and oysters is believed to be associated to direct exposure to fecal input (Silva *et al.*, 2003).

In vitro studies suggest that *Enterococcus spp*. can develop certain surviving characteristics once they are in the environment. Under nutrient deprivation, *E. faecalis* can synthesize certain proteins that make them even more resistant to other stress conditions (Hartke *et al.*, 1998). This same specie can develop a viable-but-non-culturable state (VBNC) when affected by oligotrophy and low temperatures occur at the same time (Heim *et al.*, 2002; Signoretto *et al.*, 2000). It is reported that different enterococci have the capacity of forming aggregates similar to biofilms as a defense mechanism against body macrophages (Baldassarri *et al.*, 2001; Mohamed *et al.*, 2007). This behavior was also described in vegetation (Jha *et al.*, 2005). Nutrient availability together with other physic-chemical factors are determinant for enterococci biofilm formation (Mohamed *et al.*, 2007). The formation of a mosaic-like biofilm on the

roots of *Arabidopsis thaliana* is believed to be a mechanism of survival and attachment used by three different strains of *E. faecalis*.

Overall, enterococci are considered as the best marine water pollution indicators because of their documented resistance to the characteristic conditions of seawater (USEPA, 2002). However, the whole enterococcal assemblage might be carefully used due to the different species that are not associated to a human derived pollution. The use of *E. faecalis*, *E. faecium*, and *E. hirae* is suggested as human-derived FP indicators rather than the entire enterococcal count in order to exclude enterococcal species most commonly related to a variety of non-fecal sources (Bonilla *et al.*, 2006).

1.3.4 *Enterococcus* spp. culture dependent isolation methods and media

Rapid and precise detection of FP indicators is a priority, especially for areas developed for human coastal activities. As it has been described in the previous sections of this document, *Enterococcus* spp. are FP indicators of excellence in marine environments due to their resistance to the physicochemical characteristics of this environment. Also there is a direct relationship between the density of enterococci in surface waters and an increase in swimmer-associated illnesses such as gastroenteritis (Kinzelman *et al.*, 2003).

Shortly after the first reports indicating streptococci as members of the gastrointestinal tract, it was noted that these organisms appeared to be characteristic of sewage and animal fecal wastes. It was suggested by then (year 1934) that these

bacteria were indicative of dangerous pollution (Levin *et al.*, 1975). The U.S. EPA, the World Health Organization (WHO), and the American Public Health Association (APHA) suggest some culture-dependent enterococcal isolation methods (Kay *et al.*, 2005; USEPA, 2002; APHA, 1998). These are described in many publications and have been used for water quality assessment of the coastlines of Puerto Rico (JCA, 2003).

By the mid 1950's, the use of membrane filtration for the isolation of enterococci was introduced by Slanetz *et al.* (1957) Nowadays, the two culture-dependent methods commonly used for enterococcal identification from the environment are the membrane filtration technique (Slanetz *et al.*, 1957; Griffin, 2001) and the most probable number approach (APHA, 1998). Selective media for *Enterococcus spp.* was suggested by Levin *et al.*, 1975, since the streptococcal selective media being used at the time did not differentiate between fecal streptococci and enterococci. Thus, m-Enterococcus medium was developed to be used in membrane filtration. The specificity of this media relies in the ability of enterococci to hydrolyze esculin and the inclusion of inhibitors of other organisms such as actidione (fungi) and nalidixic acid and sodium azide (Gram-negative bacteria). Triphenyl tetrazolium chloride colors enterococci to differentiate them from other streptococci based upon its reduction, and has a slight inhibitory effect on some background microorganisms (Levin *et al.*, 1975).

Many selective media for the isolation of enterococci from the environment are available. For environmental sampling, the U.S. EPA has approved the use of m-Enterococcus agar (Levin *et al.*, 1975) with modifications (Figueras *et al.*, 1996, USEPA, 2002) and Enterolert (IDEXX), among others. Enterococcosel Broth (EB)

(Becton-Dickinson) is also a selective media for enterococci. The use of EB is typically associated to antibiotic resistance analysis (Facklam *et al.*, 2002) and for enterococcal confirmation (Hartel *et al.*, 2005). The principle of enterococcosel action is described by Becton-Dickinson Company as follows:

“Enterococci and Group D streptococci hydrolyze the glycoside esculin to esculetin and dextrose. Esculetin reacts with an iron salt, ferric ammonium citrate, to form a dark brown or black complex. Oxgall is used to inhibit Gram-positive bacteria other than enterococci. Sodium azide is inhibitory for Gram-negative microorganisms”.

1.3.5 Advantages and Disadvantages of Current Methods and Media

The benefits of using modified m-E, Enterolert and EB include rapid detection of enterococci. Total enterococcal count can be obtained in 24 hours when using modified m-E and Enterolert (USEPA, 2002; Kinzelman *et al.*, 2003). Also, culture-dependent methods might present a relatively easy and inexpensive way of monitoring water quality for different environments (Figueras *et al.*, 1996; Kuntz *et al.*, 2004). Problems related to current methods include the occurrence of enterococcus-like organism (Facklam *et al.*, 2002), false positives (Hartel *et al.*, 2005), false negatives (Signoretto *et al.*, 2000), and pore clogging and/or bacterial background when using membrane filtration (Kinzelman *et al.*, 2003). The use of Enterolert, for example, have shown to be suitable for temperate and sub-tropical conditions, but not for the tropics (Hartel *et al.*, 2004). Up to 99% of false positives occurred when using that media on water and sediment samples from Puerto Rico (Hartel *et al.*, 2005). Although the current culture-dependent methodologies for the isolation of enterococci are of utmost importance to monitor areas susceptible to FP in temperate, sub-tropical, and tropical zones, isolation

methods must be carefully examined and used, due to potential inaccuracies and the effects upon the use of coastal resources.

CHAPTER 2: General Materials and Methods

2.1 Sampling methods

2.1.2 Stations

Thirteen stations from ten sites were examined at La Parguera, Lajas, Puerto Rico during this study (Table 2.1, Figure 3.1). *Thalassia testudinum* leaves and water samples were collected at four of these sites: Magueyes Island, Bird Island, Enrique Reef, and Corral Reef (Chapter 4, figure 4.1). Magueyes Island station received direct sewage effluents from a manhole that overflowed during heavy rain and/or high tides periods. Bird Island is one of the main seasonal bird rookeries where cattle egrets, yellow wing blackbirds, and pelicans rest and nest. From May to August cattle egrets predominate. Enrique Reef is frequently visited by tourists (bathers and boaters). Preliminary sampling on the water surface did not show fecal contamination. Corral Reef is an undisturbed zone, where none or minimal fecal contamination is expected. Occasionally, pelicans are observed in this site, implying a minimal chance of observing fecal input. Only seawater samples were collected in the other 6 sites (Table 2.1 and Chapter 3: Fig 3.1).

Table 2.1. Brief description of the stations sampled during this study.

Station	GPS location	Depth (±0.3 m)	General description (as reference)
Varadero	17.97553°N 67.06222°W	1.8	Direct input from the treatment plant. Close to a 5mph sign.
Crayolas (Ramp)	17.97424°N 67.05444°W	1.3	Community boat ramp
Hotels area (Villa Parguera)	17.97323°N 67.05033°W	0.5	Former waste water source from the hotel
Poblado	17.97253°N 67.64873°W	1.3	Alelí, near to El Caracol restaurant
Magueyes 1	17.97144°N 67.04533°W	1.3	Magueyes parking, by the dock. Manhole's influenced area.
Magueyes 2	17.97095°N 67.04573°W	0.6	Botero's side of Magueyes
Small creek	17.97253°N 67.0409°W	1.05	Close to a stilt house dock and the Nautical Club
Nautical Club	17.97271°N 67.03924°W	1.45	West side
Bird Island 1	17.96703°N 67.03803°W	1.15	North
Bird Island 2	17.96694°N 67.03814°W	0.75	South
Enrique 1	17.95459°N 67.04622°W	0.4	Central
Enrique 2	17.95441°N 67.04676°W	0.5	South
Corral Reef 1 *	17.94692°N 67.01523°W	2	* About 3 meters away from each other. Corral Reef was used as the control stations because minimal fecal input sources are expected in the area.
Corral Reef 2 *	17.94691°N 67.01518°W		

2.1.2 Sample collection

Date and time of sampling were recorded at each station (Table 2.1) together with water temperature, salinity, and position (GPS). A visual description of the stations for weather conditions, current movement and presence of possible fecal input sources was also recorded. Temperature was determined using an alcohol thermometer immersed close to the surface, while salinity was estimated using a VeeGee A366ATC portable refractometer. Site coordinates were taken at each sampling point with a global positioning system device (Model GPSMAP 175, Garmin International Inc., Olathe, Kansas).

Seawater (SW) samples were taken in duplicates for all of the stations using sterile stand-up Whirl-Paks (Nasco). For the seagrass (SG) study, water and *T. testudinum* samples were collected in duplicates while diving or snorkeling from Magueyes Island (1 and 2), Bird Island (1 and 2), Enrique Reef (1 and 2), and Corral Reef (1 and 2). For each site where seagrasses were sampled, SW was collected in order to be used as a blank for the water containing the SG samples. These samples were collected before the SG (at <0.3m of distance) to prevent sediment resuspension and to minimize potential enterococci blanks. About 2.5-3.5 cm of the distal portion of three to four *T. testudinum* leaves were cut and placed inside a 50mL sterile tube that was also filled with water from the sampling site. Water and *T. testudinum* samples were stored on ice packs, transported to the laboratory and processed within a period of no more than 6 hours after collection (Griffin, 2001; U.S.EPA, 2002).

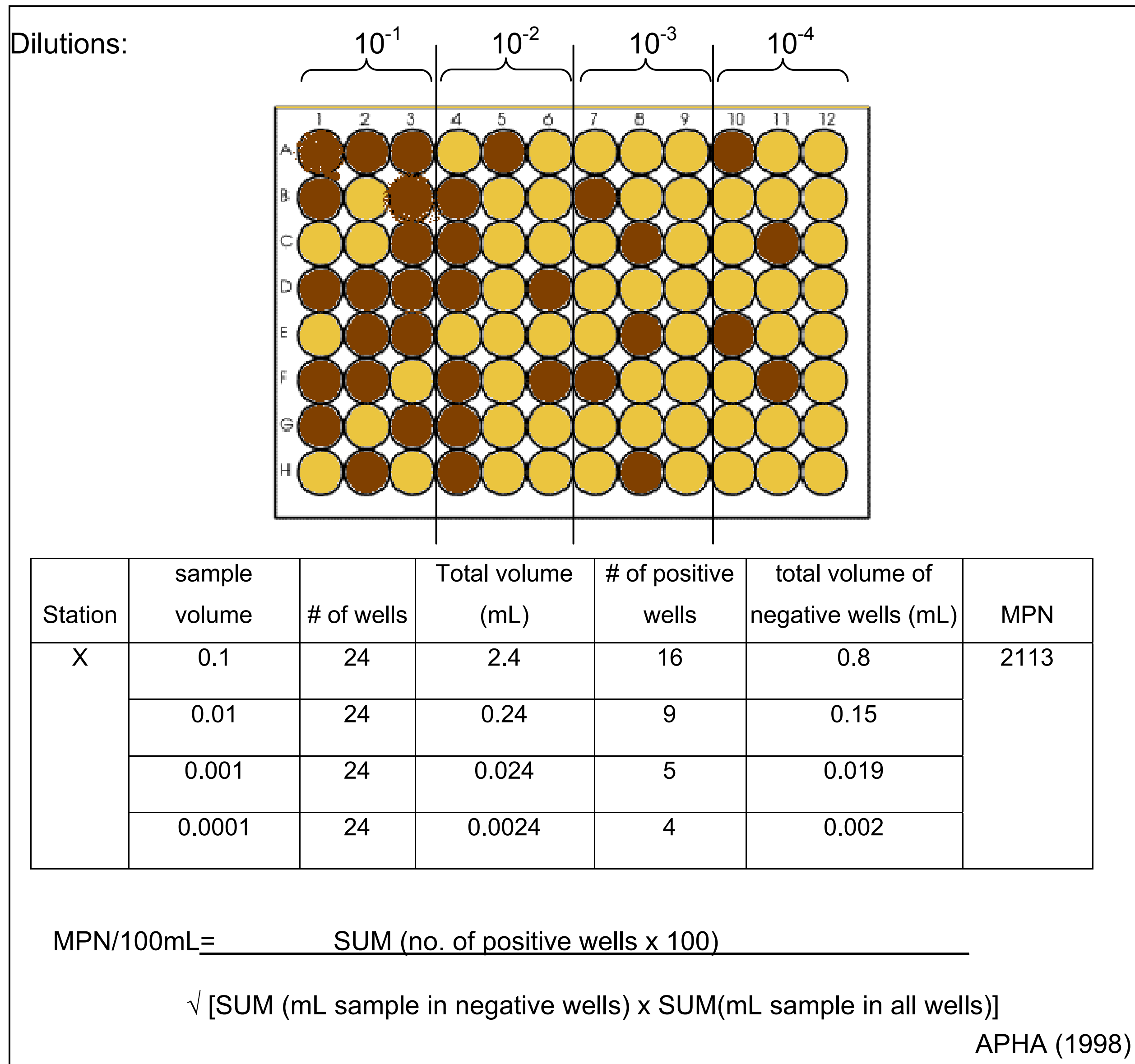
2.2 Sample processing

2.2.1 Most probable number - Serial dilutions

Three sterile tubes were supplemented with 9mL of sterile phosphate buffer (Appendix 7.2). Water sample was shaken > 30 times as suggested by U.S.EPA. One milliliter of the shaken sample was added to one of the tubes and 1/10 serial dilutions were performed using the other tubes (APHA, 1998).

2.2.2 Most probable number (MPN) using 96-well plates

The MPN procedure used during this study was adapted from APHA (1998) by using ninety six microwell plates (Corning), holding approximately 0.300mL per well (Figure 2.1), instead of the three 10 mL tubes suggested for the serial dilutions. The day before sampling each well was filled with 0.130mL of Enterococcosel Broth (Becton-Dickinson), a selective broth for enterococcal detection (Facklam *et al*., 2002). One 96-well plate was used for every sample replicate, while 24 of the 96 wells were used for each of the dilutions (Figure 2.1). Each well was added with 0.1mL of the correspondent dilution. The MPN formula was obtained from the 1998 edition of Standard Methods for the Examination of Water and Wastewater (APHA, 1998). For an example of the MPN procedure using 96-well plates see Figure 2.1. Cells were incubated for 48 hours at 40±1°C in a Precision Economy Incubator (Thermo Scientific). After incubation, dark brown wells were presumably considered as enterococci.



Station	sample volume	# of wells	Total volume (mL)	# of positive wells	total volume of negative wells (mL)	MPN
X	0.1	24	2.4	16	0.8	2113
	0.01	24	0.24	9	0.15	
	0.001	24	0.024	5	0.019	
	0.0001	24	0.0024	4	0.002	

$$\text{MPN/100mL} = \frac{\text{SUM (no. of positive wells x 100)}}{\sqrt{[\text{SUM (mL sample in negative wells) x SUM(mL sample in all wells)}]}}$$

APHA (1998)

Figure 2.1. Example on the use of 96-well plates for Most Probable Number (MPN) approach. Notice that 10^{-1} represent a non-buffer diluted sample, the dilution value is due to the volume of sampled used for the well. Dark brown spots represent Enterococcosel positive wells.

Confirmation of enterococci counts is a stepwise process. Brain Heart Infusion Agar (BHIA) (Difco) with 6.5% NaCl (Manero and Blanch, 1999) was inoculated with ten micro liters of material from every well giving a positive reaction on Enterococcosel

Broth in the first step. Media was prepared as suggested by the manufacturer, adding the equivalent amount of NaCl needed to obtain a 6.5% concentration (USEPA, 2002). Inoculated plates were incubated 24-48 hours at 37ºC. Catalase test was conducted after a secondary inoculation and regrowth on BHIA +NaCl as the final confirmation test for the presence of enterococci. The confirmed numbers of enterococci are those giving catalase (-) (no bubbling after addition of 10% hydrogen peroxide) during the final confirmation step (Cai,1999 ; Holt *et al.*, 2000).

2.2.3 Processing of *Thalassia testudinum* leaves

Back in the laboratory, each SG sample was transferred individually to 500mL sterile whirl pak. *T. testudinum* leaves were gently rubbed within each bag to dislodge cells from the phyllosphere in a way that can simulate what can happen in a real life scenario (ie. trampling, boating) . Water and suspended material were transferred to a sterile 50mL polypropylene tube, while leaves were saved for later drying and weighting. The water used as blank and the water containing the SG epiphytes was processed as described above (sections 2.2.1 and 2.2.2). The MPN for the SG samples was obtained by subtracting the corrected average MPN from the SW replicates from the MPN obtained from the water containing the SG epiphytes. The MPN from the SG were later converted to number of cells/grams of dry weight (Ott *et al.*, 2001).

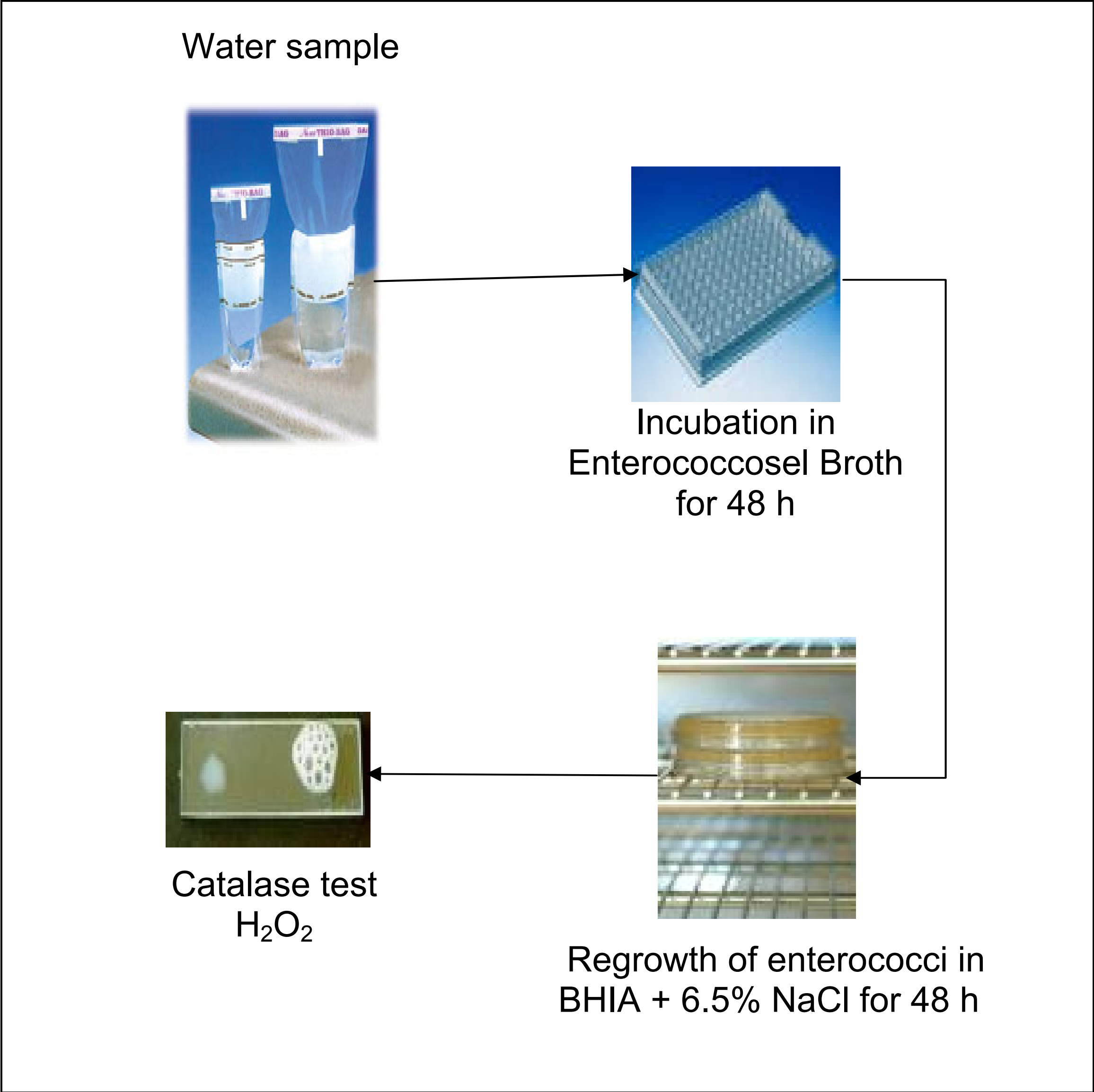


Figure 2.2. Water sample processing and enterococcal confirmation procedure diagram. Samples were collected using sterile whirl paks and processed using the most probable number approach (APHA, 1998) with Enterococcosel Broth. Isolates were confirmed as enterococci with BHIA 6.5% NaCl and catalase negative test.

CHAPTER 3: Enterococci as fecal contamination indicators at Puerto Rico's southwest shoreline: Are we using the right isolation methods?

3.1 Introduction

Enterococci have been described as the fecal contamination indicator of excellence for seawater because of their resistance to physicochemical environmental variations (Stewart *et al.*, 2007). The U.S. EPA standards for marine water quality assessment of 35cfu/100mL (geometric mean of 5 consecutive samples in a month) or 104cfu/100mL of enterococci for a single sampling (Griffin, 2001; JCA, 2003) are approved for Puerto Rico as well as the suggested isolation methods for enterococci (USEPA, 2002). Methods for FP detection must be rapid, efficient, and should be accessible to coastal communities (Kuntz *et al.*, 2004; Scott *et al.*, 2002). We tested membrane filtration with m-Enterococcus agar (Levin *et al.*, 1975) followed with esculin iron agar confirmation (Figueras *et al.*, 1996), resulting most of the times in pore clogging and/or high background bacteria. Enterolert system (Dichter, 2007) was also tested in our laboratory, resulting in a high number of false positives, in some cases up to a 99% (Hartel *et al.*, 2005). This is of concern due to the potential of using methods and standards inappropriate for tropical conditions and probably misidentifying and overestimating the numbers of enterococci when using approaches that have been developed in studies on temperate and sub-tropical conditions.

This chapter responds to the objectives of evaluating the most probable number (MPN) approach (APHA, 1998) and the necessary confirmation tests to obtain an

economic and more accurate estimate of the number of enterococci present in natural seawater samples. Ultimately, the study strives to increase access to coastal communities interested in evaluation of the quality of their water resources. The chapter also responds to the objective of identifying “hot spots” of fecal contamination at La Parguera, by considering enterococcal abundance.

3.2 Methods

Surface water samples were collected in duplicates in each of the 13 stations at La Parguera, Puerto Rico (Figure 3.1) on May 19, July 8, and October 13, 2005 and February 13, 2006. Sample processing consisted in a modification of the MPN suggested procedure (APHA, 1998) by using 96-well plates for performing the dilutions and with EB as an isolation media. Confirmation test for enterococci were performed by inoculating Enterococcosel positive bacteria into BHIA (Difco) with a concentration of 6.5% NaCl followed by the catalase test. Detailed sampling and processing methodology are described in Chapter 2: General Materials and Methods. Kruskal-Wallis one way analysis of variance on ranks followed by a pairwise multiple comparison procedure on ranks (Primer Statistical Software, 6.0) was used to compare results between MPN estimates using EB and the subsequent series of confirmatory tests needed to obtain corrected estimates of enterococci.

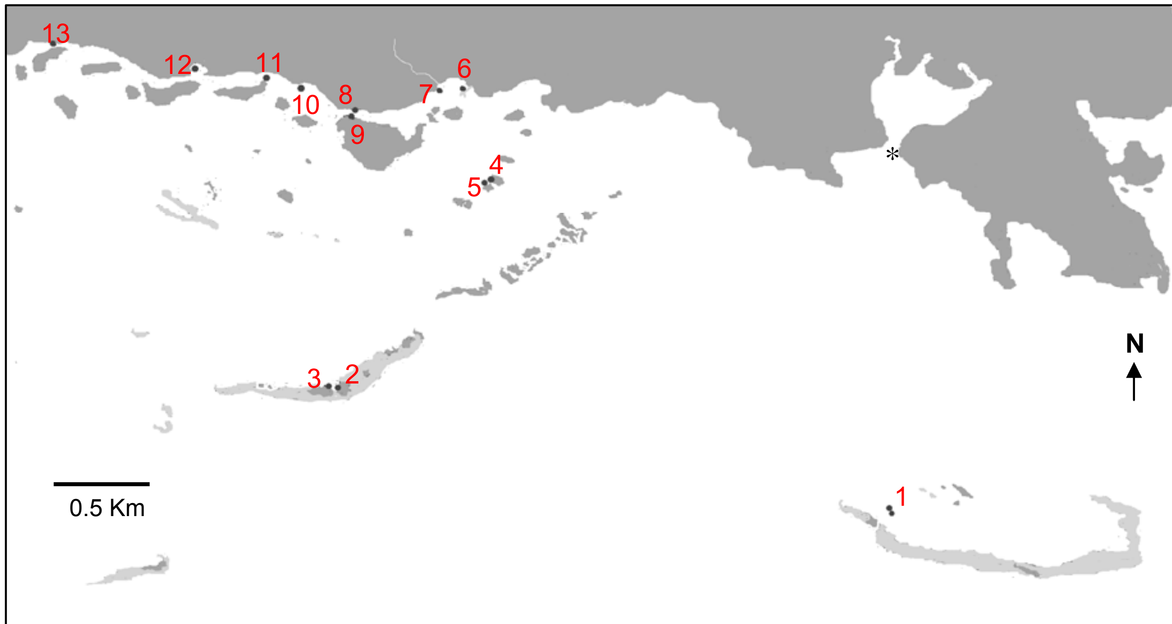


Figure 3.1. Seawater sampled stations at La Parguera, southwest Puerto Rico. The sampled stations include; (1) Corral Reef, (2, 3) Enrique Reef, (4,5) Bird Island, (6) Nautical Club, (7) Small creek, (8,9) Magueyes Island, (10) The "Poblado", (11) Hotels, (12) Boat ramp, and (13) Treatment plant. * latitude 17˚58.736' and longitude 67˚0.955.

3.3 Results

Precipitation during the sampling days was 1.27 and 0.25 mm of rain for May and October while no precipitation was recorded during the July and February sampling periods. Average precipitation during the 5 days previous to sample collection was 24.38, 7.37, 47.75, and 0.76 mm during May, July, October, and February, respectively. Average salinity and temperature among all times and stations were 34.4 PSU and 28.5˚C. Specific characteristics at each station included nesting of cattle egrets during May and July at stations 3 and 4, and manhole overflow at station 1 on October. The average enterococci counts per 100mL from EB ranged between 0-49,158 CFU, with the higher counts obtained during July (Figure 3.2).

These estimates decreased to 0-6,234 CFU/100ml after catalase test confirmation. Kruskal-Wallis ranking showed a statistically significant difference (P = <0.001) among the median values of each test (ie. EB and confirmation tests). The Tukey test indicated significant differences between EB and BHIA, and EB and catalase test results, but not between BHIA and catalase tests (Appendix 7.3). Large differences between EB-catalase (> 7 times) were observed during July in station 3 and 4, and during October in station 13 (Table 3.1).

The higher counts were reported during May and July at stations 4 and 5 (3,322 - 6,234 cfu/100mL), while the lower counts were obtained during February, when no sample exceeded the U.S. EPA limits of 104 cfu/100mL for a single sampling (Figure 3.2). Stations 2 and 3 were essentially undisturbed areas, showing only a few enterococcal isolates. The stations 1, 6, and 13 showed minimal fecal pollution during one occasion (Figure 3.2).

Table 3.1. Ratio of enterococci estimates according to the different tests for all sampling times and stations.

	May 19, 2005			**Jul 8, 2005**			**Oct 13, 2005**			**Feb 13, 2006**		
station	EB:BHI	EB:Cat	BHI:Cat	EB:BHI	EB:Cat	BHI:Cat	EB:BHI	EB:Cat	BHI:Cat	EB:BHI	EB:Cat	BHI:Cat
1	X	X	X	3.20	X	X	1.23	1.55	1.26	2.51	5.20	2.07
2	X	X	X	1.60	4.19	2.62	1.00	1.00	1.00	x	X	x
3	X	X	X	7.38	7.38	1.00	2.00	X	X	5.03	X	x
4	1.25	1.25	1.00	7.30	10.56	1.45	1.24	2.10	1.70	3.03	X	x
5	1.23	1.23	1.00	1.91	2.87	1.50	1.33	4.00	3.00	1.50	1.50	1.00
6	1.21	1.21	1.00	1.51	1.51	1.00	1.16	1.52	1.30	x	X	x
7	1.26	1.26	1.00	1.00	1.00	1.00	1.59	2.22	1.40	4.03	X	x
8	1.50	1.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.00	X	x
9	1.56	1.75	1.12	1.05	1.05	1.00	2.19	2.19	1.00	6.14	X	x
13	2.06	2.06	1.00	2.00	2.00	1.00	1.06	2.83	2.67	1.00	x	x
11	5.14	5.14	1.00	1.00	1.00	1.00	1.09	1.30	1.19	x	x	x
12	1.15	1.15	1.00	3.03	3.03	1.00	1.05	2.12	2.02	1.00	x	x
13	1.60	1.60	1.00	X	X	X	2.08	30.60	14.72	x	x	x

Cat (catalase test), x= no enterococci.

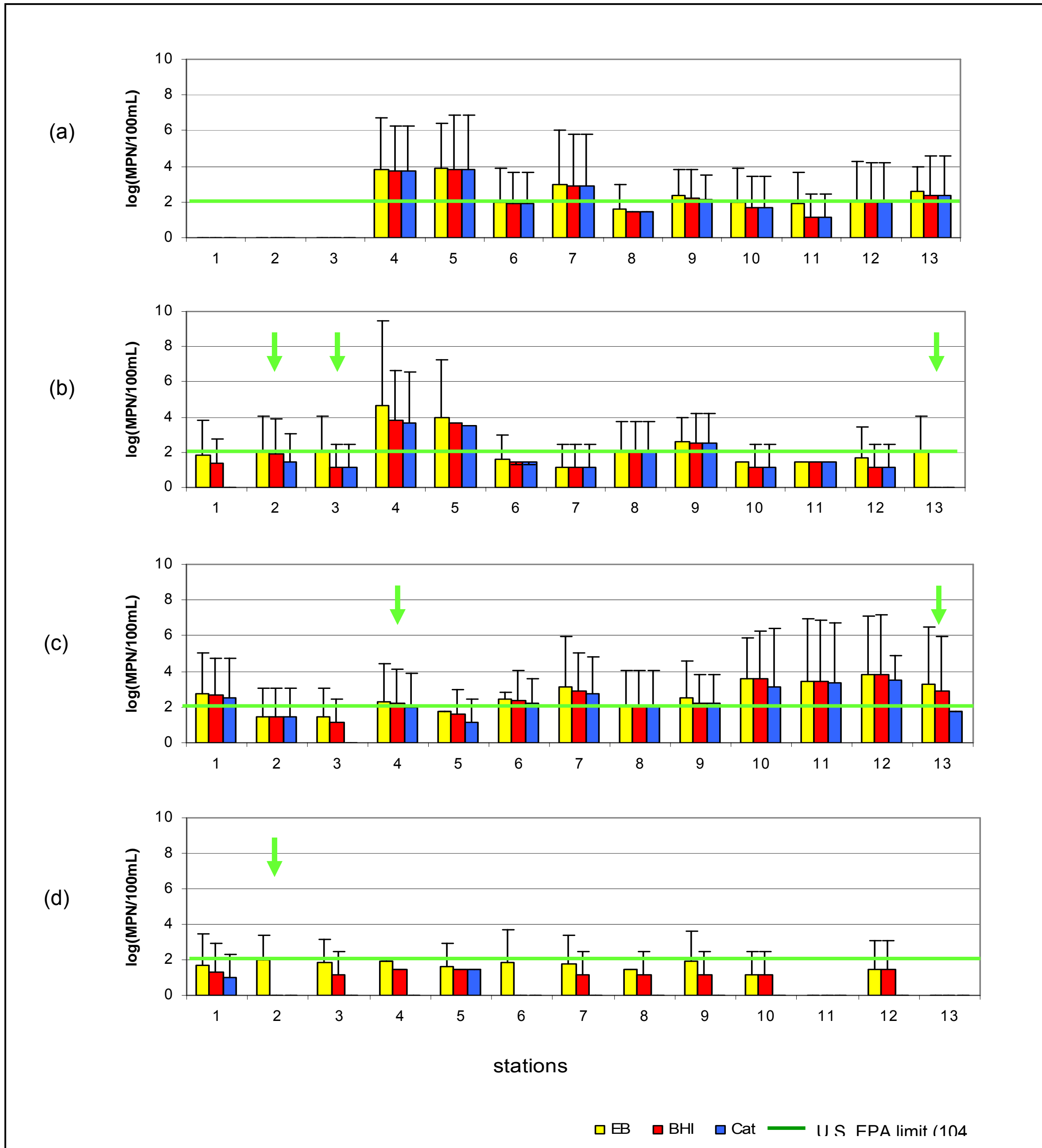


Figure 3.2. Enterococcal confirmation procedure from all sampled stations and times at La Parguera. Periods of sample collection included (a) May 19, 2005, (b) July 8, 2005, (c) October 13, 2005 and (d) February 13, 2006. The U.S. EPA limit indicates the logarithm of the one day of sample standard for marine waters (104 cfu/100mL). Light- green arrows denote the stations at which fecal pollution was observed previous to the confirmation procedure. Results are expressed as the logarithm of the EB, and BHIA and catalase-corrected MPN from all the stations and sampling periods.

3.4 Discussion

Enterococcosel Broth is typically used as a primary isolation media for antibiotic resistance assays (Facklam *et al.*, 2002) and as a confirmation step when isolating enterococci from the environment (Hartel *et al.*, 2004). In this study, EB was tested as the primary isolation media from marine environments. Results from this study suggest that when using the MPN-EB methodology, confirmation tests should be performed at least to BHIA- 6.5% NaCl, because of the no significant difference reported between BHIA-6.5% NaCl and catalase. This suggests that confirmation steps might be inverted by performing catalase examination before the BHIA 6.5% NaCl confirmation, saving time in a situation that requires fast decisions and action for the proper control of a FP event, and also saving materials by confirming only the catalase negative bacteria instead of the total esculin positive bacteria. This has to be further tested in order to assess the viability and precision of using the catalase examination as a primary confirmation test.

Although differences between EB and catalase confirmation were observed, large drops on enterococci numbers after correction (>5 X) were seen only in a few occasions, including May at station 11, in October at station 13, and during July at station 3 and at station 4 when cattle egrets were present. The corrected values at station 4 remained >40X higher than the established limit. In contrast, the uncorrected values from stations 3 and 13 (122 MPN/100ml and 1775 MPN/100mL, respectively) were higher than the established limit while the corrected value for both stations was bellow the U.S. EPA limit (Figure 3.2). The tendency of apparent FP was also observed

in other stations (Figure 3.2). These included also the touristic area of Enrique Reef during July (station 2) and February (station 3). High numbers of enterococci can be indicative of possible outbreaks (Griffin, 2001) and promote possible beach closings and warnings from places where no real FP was occurring.

The reason why a high number of false positives were observed only in a few stations is unclear, but the occurrence of these false positive numbers in areas of higher potential for the presence of wild warm-blooded animals support the possibility of the presence of other enteric bacteria. Some streptococci associated with fecal material, such as *Streptococcus bovis*, are able to hydrolyze esculin, but do not tolerate NaCl in a concentration of 6.5% (Holt *et al.*, 2000). *Streptococcus bovis* is mainly catalase negative and is commonly found in the alimentary tract of cows, sheep, and other ruminants (Ghali *et al.*, 2004).

In general, FP in La Parguera was variable among stations and times. Fecal pollution incidence was higher during time of precipitation. During October 2005, when the highest precipitation was recorded, 69% of the sampled stations exhibited FP. May and July, 2005 also presented a similar pattern, in which the potential of FP increased with precipitation (46% and 31% of the stations, respectively). During the dry season (February, 2006), enterococci were detected in only 2 of the stations sampled but after correction using catalase testing, no FP was observed. These results are in agreement to those of Harwood (2007) and Lipp *et al.*, 2001, that indicated higher abundance of enterococci during higher precipitation and stormy conditions.

Considering the environmental setting found at each station, the high enterococcal numbers on May and July at station 3 were probably due to the nesting of cattle egrets. The treatment plant area (station 13) did not seem to be the main source of enterococci in the area. Previous works in La Parguera showed similar observations. In a study that included enterococci as well as other biological and chemical indicators of contamination, no significant differences between the sewage treatment facility and the other stations, including off-shore stations (Ramirez-Toro *et al.*, 2005). Because of the limited details available from that study, it is not known if enterococcal confirmation was performed. Some of the stations sampled in the present study showed FP, but not due to enterococci as indicated by catalase testing (Figure 3.2).

In conclusion, a re-evaluation of the methods used for *Enterococcus* spp. isolation and consequently the standards used for our tropical coasts is suggested. Limitations of current methods such as membrane filtration include interference by pore clogging and overgrowth of background bacteria (Kinzelman *et al.*, 2003). This is also true for zones with high turbidity. The approach presented here, using a MPN approach with EB on 96-well plates is economically accessible, but still needs to be improved. It might be complemented with microscopic observations to evaluate cells shapes (i.e. clustering vs. chains) and enterococcal speciation. Speciation might be an option to determine contamination origin and so if the enterococci present are FP indicators (i.e. *Enterococcus faecalis*, *E. faecium*, *E. hirae*), are an environmental contribution or even if there are still false positives (ie. enterococcus-like bacteria, Chapter 4). Studies on antibiotic resistance patterns (Harwoood et al., 2000), molecular markers of specific species such as *E. faecium* (Scott et al., 2005), among others, may be suitable as a last

confirmation step and could provide further testing of the described approach. Also, microbial source tracking approaches should be considered in order to identify the main source of FP and therefore prevent possible feces related illnesses for humans and the environment. Enterococci should be studied for this concern; however, studies on specificity should be performed.

CHAPTER 4: Enterococcus spp. diversity and the use of enterococci as an indicator of fecal contamination source in a tropical marine environment.

4.1 Introduction

Enterococcus spp. are used worldwide as FP indicators for fresh and sea water environments (Bordalo *et al.*, 2002; Figueras *et al.*, 1996; Kinzelman *et al.*, 2003; USEPA, 2002). The apparent host-specificity (Baele *et al.*, 2002; De Graef *et al.*, 2003; De Vaux *et al.*, 1998; Naser *et al.*, 2005; Ŝvec *et al.*, 2005b) and variable range of abundance of certain enterococci in different hosts suggest their use as source trackers (Kuntz *et al.*, 2004; Wheeler *et al.*, 2002). Microbial source tracking (MST) is defined as matching a FP indicator with a particular animal source, such as human, farm animals, and wildlife, with the intention of providing the tools for an adequate management of a FP event (Harwood, 2007; Scott *et al.*, 2002; Stewart *et al.*, 2007).

Certain human related enterococci such as *E. faecalis*, *E. faecium,* and *E. hirae* (Bonilla *et al.*, 2006; Kuntz *et al.*, 2004) have been reported to occur and persist in environments such as sediments and vegetation (Hartel *et al.*, 2004; Müller *et al.*, 2001; Mundt, 1963; Ott *et al.*, 2001; Whitman *et al.*, 2003, 2005). Other enterococci have been reported to naturally occur in non-warm blooded host sources such as seawater (Ŝvec *et al.*, 2005a) and foliage (Lindow and Brandl, 2003; Müller *et al.*, 2001). During this study, *Thalassia testudinum*, one of the most abundant seagrass species of the Caribbean (García-Ríos, 2001), was evaluated as a potential source and reservoir of warm-blooded associated and naturally occurring enterococci by sampling in different sites, and by direct inoculation of *E. faecalis* into this SG. The proximity of this seagrass

to the shore adds a potential susceptibility of being affected by the input of a variety of contaminants, thus increasing the opportunity of colonization by bacteria and epiphytes (Newell, 1981). During this work the use of enterococci for MST at tropical sites was examined by analyzing their spatial and temporal patterns of diversity and the enterococcal species association to areas with potential human and other warm-blooded animals derived FP. In addition, enterococcal survival in *T. testudinum* was evaluated. Understanding enterococcal diversity and its occurrence in different warm-blooded, and natural hosts and environmental conditions will hopefully lead to better decisions in terms of the pertinent actions to take during FP events in the tropics.

4.2 Methods

4.2.1 Sampling sites

The study was conducted at La Parguera Natural Reserve (17˚ 57'N, 67˚ 02'W), southwest Puerto Rico (Detrés *et al.*, 2001). La Parguera is characterized by its dry weather and the rich variety of marine ecosystems, including mangroves, seagrasses, coral reefs, and bioluminescent bays, which makes it attractive for tourism (Boshetti-Aponte, 2002). Fishing, boating, and stilt houses along the coast are common. About 66 bird species have been previously reported (Ríos, 1984). These characteristics make the area suitable to study enterococcal species diversity from different hosts and environmental conditions.

4.2.1 Sample collection

Seawater (SW) and SG leaves were collected at eight stations (Figure 4.1) during May 19, July 8, and October 14, 2005 and February 13, 2006. Stations 1 and 2 are located near Magueyes Island, where the Marine Sciences Department laboratories (University of Puerto Rico) are located. Station 1 (17.97144°N, 67.04533°W) is near a parking area and receives intermittent pulses of used waters due to its proximity to an overflowing manhole. In addition, this station is affected by surface run-off during episodic rains. Station 2 (17.97095°N, 67.04573°W), is located at the opposite shore of the waterway, in the main access to Magueyes Island, where sediment resuspension results from the shuttle boat. Because of the accessibility of the station (i.e. no boat required), samples were collected at a different time than those of station 1 (4-5 hours later), what permitted to process these samples closer to the collection.

Stations 3 and 4 are located immediately downcurrent to a bird rookery where cattle egrets, yellow wing blackbirds and brown pelicans rest and nest seasonally. Station 3 (17.96703°N, 67.03803°W) is northward while station 4 (17.96694°N, 67.03814°W) is located southward of the current influx. Stations 5 and 6 are located in the backreef of Enrique Reef and are frequented by tourists. The two stations (17.95459°N, 67.04622°W and 17.95441°N, 67.04676°W) are located northeast and southwest respectively. Stations 7 and 8 are located by Corral Reef (17.94692°N, 67.01523°W) and were included as control stations. Data on precipitation was obtained from the USGS (station # USGS 50128900 Lago Loco at Damsite NR Yauco, PR). Samples

were processed using the most probable number (MPN) technique with Enterococcosel Broth (EB) and confirmed by spreading over Brain-heart infusion agar (BHIA) - 6.5% NaCl and by the catalase test. Sample collection and processing are fully described in the Chapter 2 of this document.

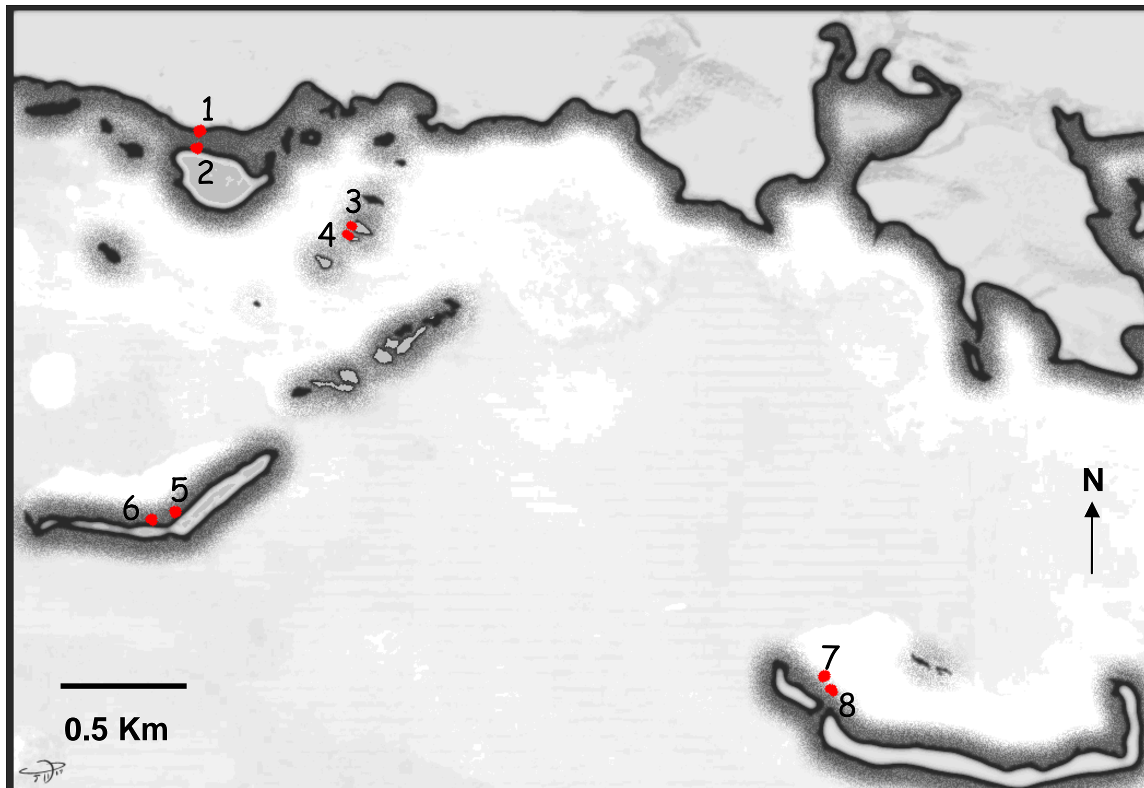


Figure 4.1. Seawater and *Thalassia testudinum* sampled stations at La Parguera (17˚ 57'N, 67˚ 02'W). Stations 1 and 2 were located in Magueyes Island, which holds marine sciences laboratories; stations 3 and 4 were located in what is popularly called Bird Island because of the seasonal occurrence of different bird species. Station 5 and 6 were in the backreef of Cayo Enrique, a touristic area, and station 7 and 8 are in Corral Reef, the control station.

4.2.2 Species composition.

In order to determine the species composition of the enterococcal community at each station and time, catalase negative microbes from SW and SG samples were tested for

their capacity to use arginine and ferment mannitol, methyl–α–D-glucopyranoside, sorbose, ribose, arabinose, and sucrose (Manero and Blanch 1999; Carvalho *et al*., 1998; Facklam *et al*., 2002). *Enterococcus faecalis* (ATCC 29212), *E. faecium* (ATCC 35667), *E. gallinarum* (ATCC 49608), *E. durans* (ATCC 6056), and *E. avium* (ATCC 14025) were used to evaluate the accuracy of the speciation approach.

The total enterococcal species present at each time and station is defined as species richness (r). The reciprocal of Simpson's diversity index was applied for biodiversity assessment of Enterococcus populations and was calculated for each station and time as $D=1/\Sigma\ (N_i/N)^2$, where N_i is the total number of organisms of a particular species and N is the total number of enterococci from the particular station for each time. Simpson's equitability was calculated as $E_D=D/r$ and was used to observe if there was a dominant species in the sample (Anderson *et al*., 2006; Cox and Gilmore, 2007; Simpson, 1949). Both were calculated using Microsoft Excel.

4.2.3 Direct inoculation of enterococci into *Thalassia testudinum*.

Enterococcus faecalis (ATCC 29212), a species isolated from human urine, was directly inoculated onto the surrounding water of *T. testudinum*, using a novel approach, to corroborate if this SG species acts as an underwater reservoir for enterococci of human origin. The study was conducted (1) *in situ* at Corral Reef (station 1 on figure 3.1) and (2) *in vitro*, using fish tanks as incubation chambers. Corral Reef (17.9469°N, 67.0152°W) presents low or none direct influence of fecal input sources. Previous

sampling presented enterococcal numbers below the U.S. EPA limits in SW and low numbers on SG samples.

Enterococcus faecalis (ATCC 29212) was inoculated in BHIB and then preserved on glycerol-phosphate buffer in a -80°C freezer. Prior to the direct inoculation assay, *E. faecalis* was recovered from -80°C with Enterococcosel Broth and incubated at 39 ±1°C for 48 h in a Precision Economy Incubator (Thermo Scientific). For both direct inoculation assays, *E. faecalis* was pre-adapted to the characteristic salinity and temperature of the experimental area by incubating on BHIA with an addition of 3.4% NaCl at 39 ±1°C for 48 h. On the day of the direct inoculation on SG, one colony was transferred to each of three sterile tubes containing 50mL of BHIB 3.4% NaCl and were incubated at 29 ±1°C for 6 h (pre-determined log phase of our strain on the medium used). To assure a similar number of enterococci on each of the incubations, the three batches were mixed in a sterile 500 mL bottle and returned to the three 50 mL tubes. Finally, the content of one of the three tubes was used be inoculated into one of three boxed areas (see below) in Corral Reef or in each of the incubation chambers used during the second assay.

Inoculation in Corral Reef was conducted during August 17-20, 2005 and March 27-30, 2006, starting approximately at 9:00AM in both cases. Three clear polyethylene plastic chambers, holding 17.2 L each, were placed upside down over SG at 5 m intervals in a north-south direction. A 60 mL sterile syringe was used to transfer the bacterial inoculum from the 50 mL tubes into each chamber by means of an opening fitting the syringe tip. A fourth control chamber was placed 5 m upcurrent to the east to

minimize the potential for enterococci contamination from the experimental chambers. The chambers were left for 6 h, in order to allow for the establishment of enterococci on the SG leaves. Water and SG samples were collected 3 - 4 days and immediately previous to inoculation, just after chamber removal (T6), and 24 and 48 h after inoculation.

The direct inoculation of enterococci on incubation chambers with *Thalassia testudinum* was conducted in four 25 L fish tanks with a 7 cm deep calcareous sand layer. Healthy plants of the seagrass were collected in two locations in La Parguera: Monsio José Bay (17˚57.060' N, 67˚5.530' W) and from the west side of Magueyes Island (17˚58.155' N, 67˚2.771' W). The roots of 6-8 plants with an average of 0.20 m high were tied to each of 24 30 cm PVC tube segments, six from which were anchored in each tank using sand to cover seagrass roots. Seagrasses were cut afterwards to a height of 12 ± 4 cm. To evaluate the possible relation of *T. testudinum* epiphytes with enterococcal attachment, the surface of the leaves of SG destined to two tanks were aseptically cleaned, wearing gloves and removing all the visible matter from the surface of the leaves. After transplantation, SG were acclimated for 11 days before inoculation. During this period, each tank was maintained with flowing SW and under sunlight to maintain salinity and temperature as close to natural conditions as possible. The SW flow was approximately 1.5 L min^{-1} and was evenly distributed using a hose with several punctures that was laying on the bottom. The incubation chambers designation was as follows:

Chamber 1: Epiphyte cleaned SG, no inoculum (control)

Chamber 2: Epiphyte cleaned SG, *E. faecalis* addition

Chamber 3: Epiphytes, no inoculum (control)

Chamber 4: Epiphytes, *E. faecalis* addition

The SW flux was suspended a few minutes before *E. faecalis* inoculation and re-established after 6 h (T6 samples) similarly to what was performed in the *in situ* incubations. Water and SG samples were taken in triplicates before and after inoculation and 3, 6, 12, 18, and 24 h afterwards. Seawater and SG samples were collected using sterile Whirl Paks and 50 mL sterile tubes, respectively. Samples were processed using the MPN-EB approach described in Chapter 2.

In a second approach, *E. faecalis* (ATCC 29212) was inoculated on each of two 10 L fish tanks without SG in order to estimate the effect of dilution when inoculating a higher number of cells in SW. Incubations started overnight at 12:00AM. Samples were collected and processed as previously described in this document.

4.3 Results

4.3.1 Spatial and temporal environmental conditions.

The average temperature and salinity for all stations and times were 28.4˚C and 34.9 PSU. Precipitation during the sampling days was 1.27 and 0.25 mm of rain for May and October while no precipitation was recorded during the July and February sampling periods. Average precipitation 5 days before sample was 24.38, 7.37, 47.75, and 0.76 mm during May, July, October, and February, respectively. Station 1 received

intermittent overflows from a nearby manhole. During May through July stations 3 and 4 were adjacent to a nesting site of cattle egrets.

4.3.2 Enterococcal occurrence and diversity.

After pooling the data from all times and stations for SW and SG assays, about 22 enterococcal species were collected from La Parguera. These included representatives from groups II, III, IV, and V, used for classification of enterococci (Appendix 7.1). Enterococcus-like bacteria (those enterococci that were not able to be classified using the substrates used in this study) were grouped together. Also some enterococcal isolates were grouped as GIII and GV since additional biochemical tests would have been needed to separate them from other species of the same group (i.e. *E. durans*). No members of the GI enterococci (*E. avium, E. malodoratus, E. raffinosus, E. pallens, E. pseudoavium, E. saccharolyticus, E.gilvus, E. phoeniculicola*, and *E. devriesei)* were found. All species, with the exception of *E. cannis* and *E. herminiensis,* were isolated from SG at least once. Meanwhile, three of the isolated species, *E. casseliflavus*, *E. mundtii*, and *E. columbae* did not occur on SW. In general, all species tend to be more abundant on SG (Figure 4.3). However, there wasn't a clear pattern in the species dominance with relation to locations or time on either SG or SW (Figures 4.3, 4.4).

Enterococcal richness, diversity, and equitability were calculated for each station and time (Table 4.1). According to the reciprocal of Simpson's index, Bird Island (stations 3 and 4) and Magueyes (stations 1 and 2) showed the highest diversity of enterococci (Table 4.1) in both SG and SW relative to Enrique (stations 5 and 6) and El

Corral (stations 7 and 8). In some cases the equitability (E_D) at these stations was <0.5, indicating that there was species dominance within the enterococcal community. Enterococcal biodiversity was highly variable with respect to time and stations. Enterococci were practically absent in the off-shore stations with the exception of station 6 during May 2005 (496 cells/g of *E. hirae* and enterococcus-like bacteria on SG) and Corral Reef during October 2005 (>306 cfu/100mL of *E. faecium* (II and III), *E. gallinarum* (II, V), *E. hermaniensis*, *E. villorum*, *E. solitarous*, *E. asini*, GV, *E. faecalis* V, *E. solitarous*, *E. columbae*, GV enterococci, and enterococcus-like bacteria in SW and up to 2130 cells/g of dry weight on SG). *Enterococcus* spp. were absent or reached their lowest diversity in all stations during the month of February 2006.

Table 4.1. Diversity Indices of enterococci in all stations and periods.

SG	**May**			**Jul**			**Oct**			**Feb**		
station	R	D	E_D	R	D	E_D	r	D	E_D	r	D	E_D
1	7	5.07	0.72	4	3.39	0.85	8	4.62	0.58	x	x	x
2	10	5.57	0.56	5	3.51	0.70	8	4.18	0.52	2	2.00	1.00
3	10	4.52	0.45	9	3.93	0.44	4	3.33	0.83	x	x	x
4	7	4.79	0.68	10	6.60	0.66	4	2.91	0.73	2	1.99	1.00
5	X	x	X	X	X	X	x	x	x	x	x	x
6	2	1.60	0.80	X	X	X	x	x	x	x	x	x
7	X	x	X	X	X	X	4	2.91	0.73	x	x	x
8	X	x	X	X	X	X	5	2.61	0.52	x	x	x
SW	**May**			**Jul**			**Oct**			**Feb**		
station	R	D	E_D	R	D	E_D	r	D	E_D	r	D	E_D
1	1	1.00	1.00	3	2.88	0.96	5	4.45	0.89	x	x	x
2	5	4.26	0.85	1	1.00	1.00	4	3.52	0.88	x	x	x
3	7	4.52	0.65	12	4.21	0.35	3	2.57	0.86	x	x	x
4	8	2.86	0.36	11	3.59	0.33	1	1.00	1.00	1	1.00	1.00
5	X	x	x	X	X	X	2	2.00	1.00	x	x	x
6	X	x	x	X	X	X	x	x	x	x	x	x
7	X	x	x	X	X	X	3	2.33	0.78	x	x	x
8	X	x	x	X	X	X	5	3.60	0.72	x	x	x

Species richness (r), Simpsons Diversity Index (D), and equitability (E_D) are presented for seagrass (SG) and seawater (SW) samples. The (x) represents stations where no enterococci were isolated. The richness indicates the higher possible value of diversity for each station/time.

4.3.3 Occurrence of enterocci associated with warm-blooded animals.

Enterococcus gallinarum, *E. faecium* (II, III), *E. faecalis* II, *E. hirae*, and *E. villorum* were the most common enterococci in both, SG and SW samples (Figure 3.2). Both groups *of E. faecium* were generally present. *Enterococcus faecalis* GII and *E. hirae* were principally isolated from SG (Figure 3.2). *Enterococcus faecalis* GII was more frequent in areas associated to bird rookeries. *Enterococcus faecalis* GV was isolated only in two occasions: on SW from Enrique Reef (14 cfu/100mL) during October and on SG from the control station (98 cells/g) during the low precipitation period (February 2006). *Enterococcus hirae* was the only species isolated during all sampling periods although *Enterococcus gallinarum* was in general the most abundant, especially on stations 3 and 4 on July, during bird rookering. Proportions of these species were temporally and spatially variable (Figure 3.3).

4.3.4 Other *Enterococcus* spp. and enterococcus-like bacteria.

Plant associated enterococci, such as *E. casseliflavus* and *E. mundtii*, were observed occasionally only on the SG samples. *Enterococcus casseliflavus* occurred during May and July at station 3 (5% of 31092 cells/g and 1% of 44225 cells/g, respectively) while *E. mundtii* was found during May in station 3 (10% of 31092 cell/g) and October in stations 1 and 3 (10% of 1329 cells/g and 20% of 963 cells/g). Enterococci GIII were present in areas where human derived FP from animal sources was expected (ie. stations 1, 2, 3, and 4). The biochemical examination used during this study (Appendix 7.1) was not able to differentiate between *E. durans, Ent faecalis* GIII,

E. silesiacus, and *E. ratti*. Enterococcus-like species were highly abundant in both SW and SG samples (Figures 3.2 and 3.3), been in some cases more dominant than identified enterococci.

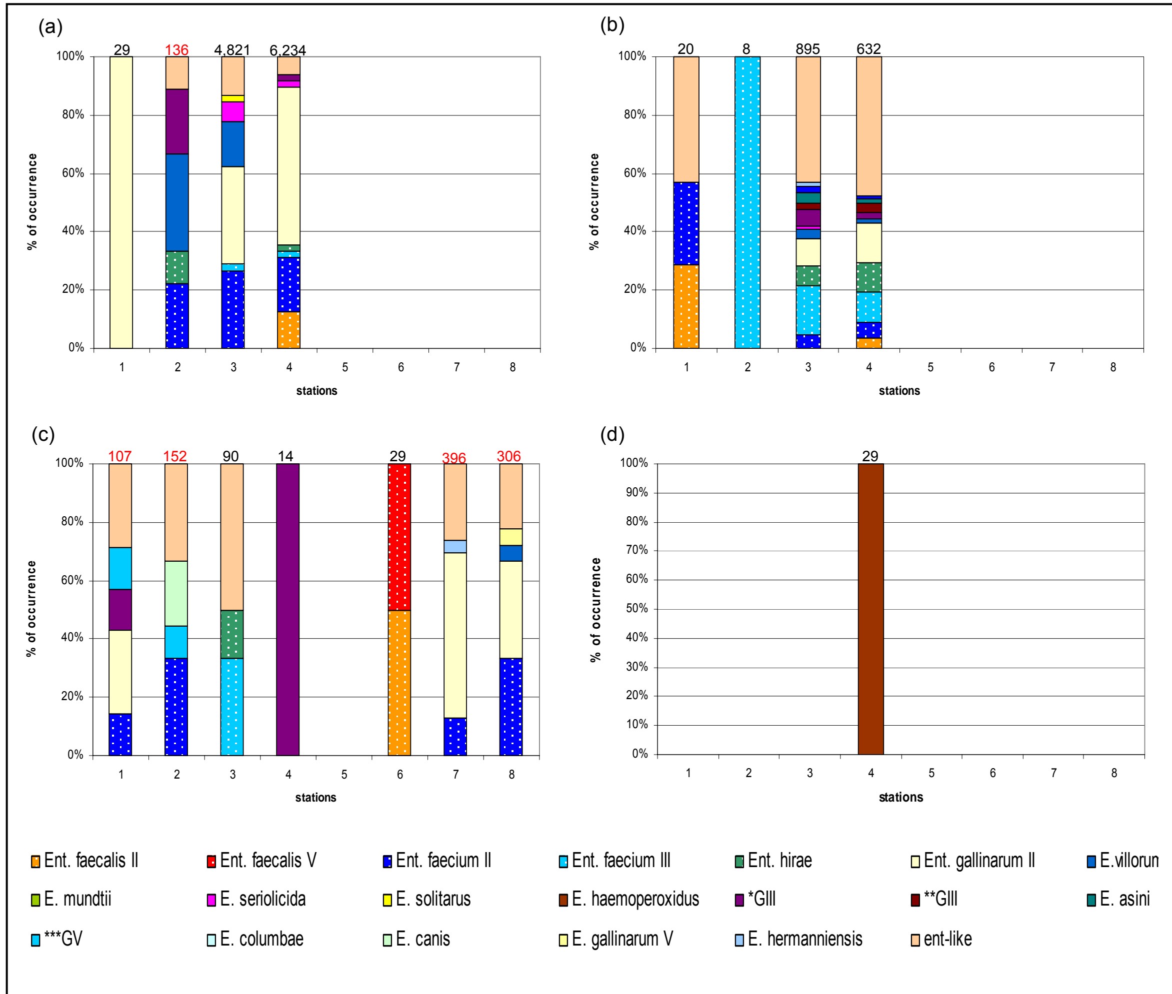


Figure 4.2. Relative abundance and species composition of *Enterococcus* spp. from seawater (SW) samples in all stations and times. Sampling dates included (a) May 19, 2005, (b) July 8, 2005, (c) October 14, 2005 and (d) February 13, 2006 in La Parguera. Stations included in this study were Magueyes Island (1 and 2), Bird Island (3 and 4), Enrique Reef (5 and 6), and Corral Reef (7 and 8). *GIII = *E. durans, E. ratti, E. faecalis* III or *E. silesiacus*; **GIII= *E. dispar* or *E. canintestini*; ***GV = *E. moraviensis E. casseliflavus* V. Dotted columns indicate FP related enterococci. The number in the top of the columns refers to total MPN/100mL of SW at each station. Numbers in red indicate stations with total enterococcal counts higher than the the U.S. EPA limit, but with total human FP indicators below the limit.

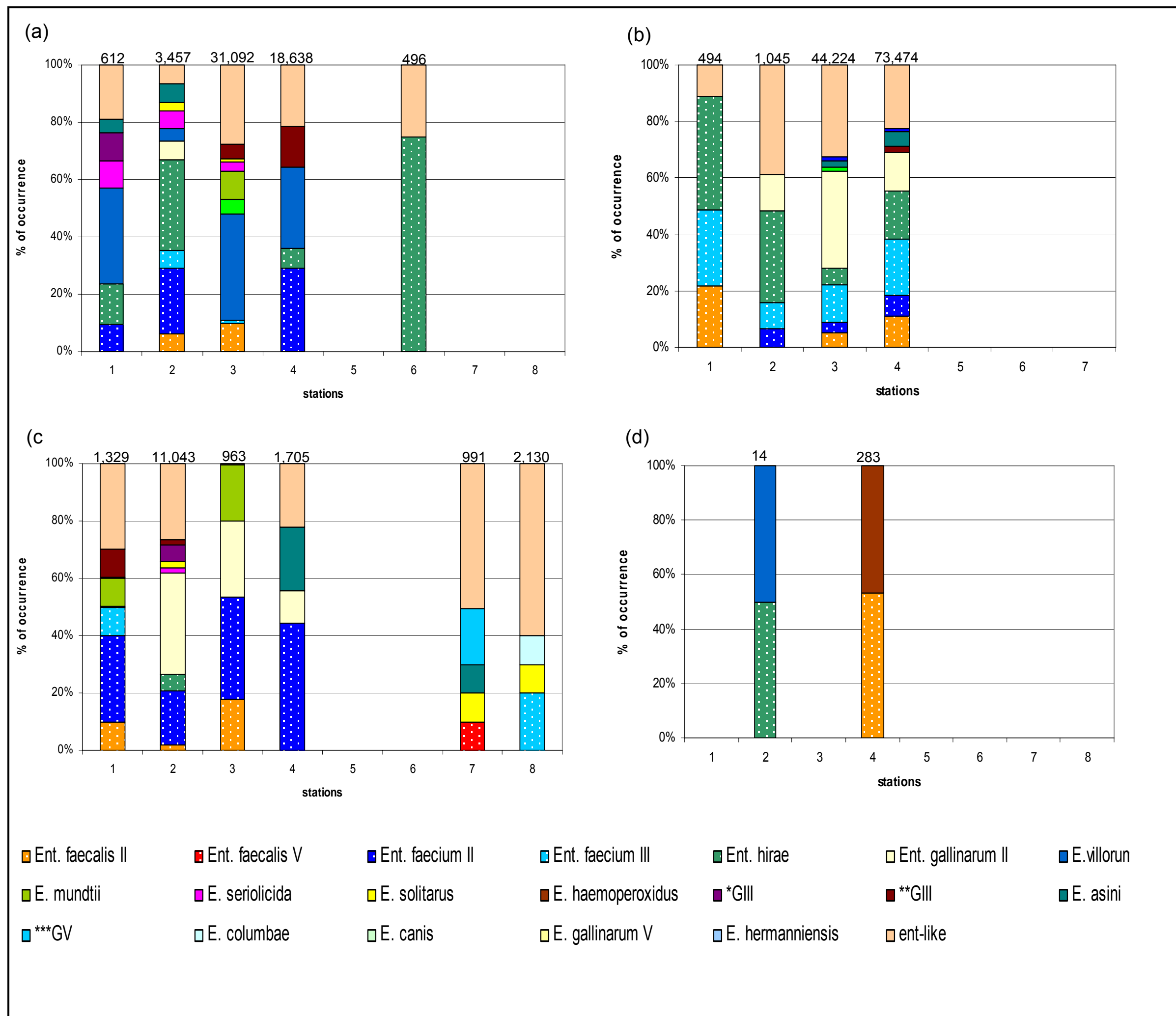


Figure 4.3. Relative abundance and species composition of *Enterococcus* spp. from seagrass (SG) samples in all stations and times. Sampling dates included (a) May 19, 2005, (b) July 8, 2005, (c) October 14, 2005 and (d) February 13, 2006 in La Parguera. Stations sampled in this study were Magueyes Island (1 and 2), Bird Island (3 and 4), Enrique Reef (5 and 6), and Corral Reef (7 and 8). *GIII = *E. durans, E. ratti, E. faecalis* III or *E. silesiacus*; **GIII= *E. dispar* or *E. canintestini*; ***GV = *E. moraviensis E. casseliflavus* V. Dotted columns indicate FP related enterococci. The number in the top of the columns refers to the cells/g of dry wt of SG at each station.

4.3.5 Direct inoculation assay.

Bacterial counts previous to the inoculation were calculated as 1.4 x 10^7 CFU/100mL during August and as 1.3 X 10^8 CFU/100mL. No attachment was observed in Corral Reef seagrasses and a rapid disappearance in both, Corral Reef and fish

tanks assays. During August in Corral Reef, only 5 cfu/100mL of enterococci were detected after chamber removal (T6) on SW samples, while no detection was evident during March. Seagrasses harbored enterococci but the numbers were relatively low (Figure 4.4).

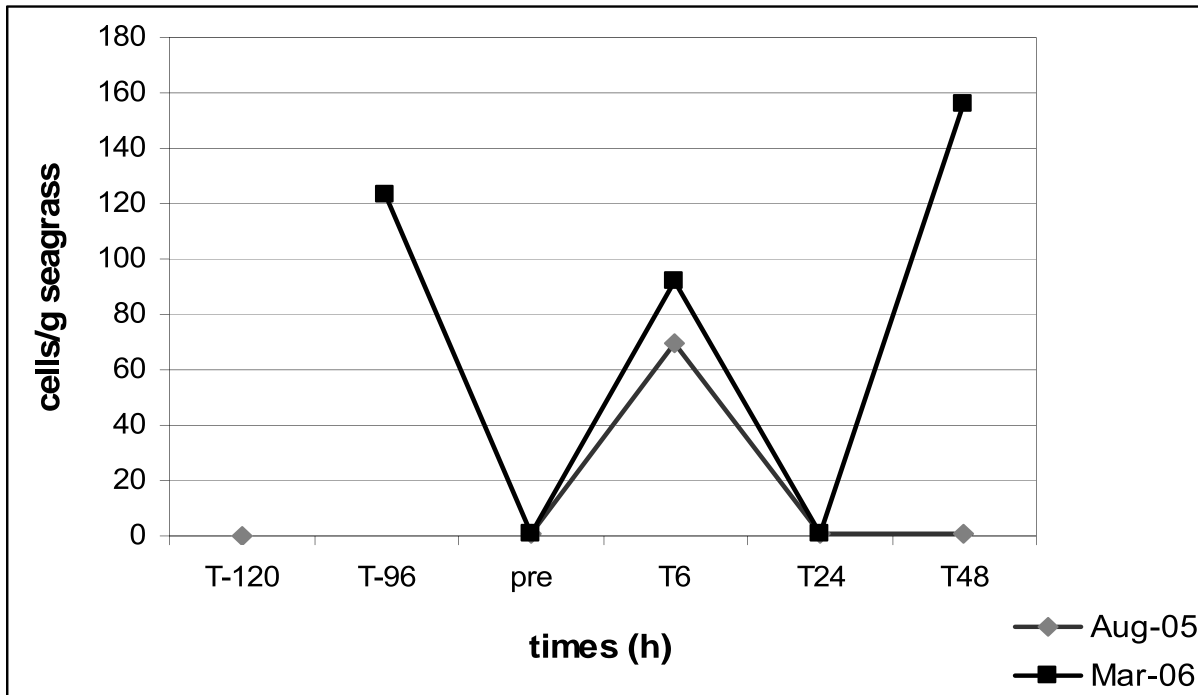


Figure 4.4. *Enterococcus faecalis* (ATCC 29212) persistence on seagrasses when inoculated into boxed areas at Corral Reef on August, 2005 and March, 2006. Chambers were removed on T6. pre= few minutes before the inoculation

Inoculation in running seawater tanks confirmed that enterococci were able to grow when water flow was suspended, but disappeared rapidly and could not keep up due to high dilution rate (Figures 4.5 and 4.6). Attachment to the SG was not observed during the same experiment. However, the abundance of enterococci was higher in the

presence of epiphytes during the pre-dilution period in the controlled environment of the incubation chambers (Figure 4.5)

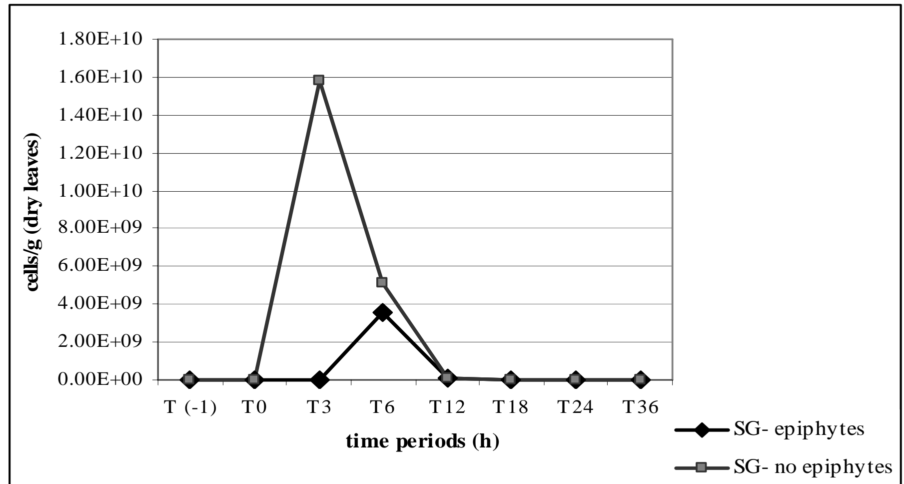


Figure 4.5. Differential survival of *E. faecalis* (ATCC 29212) on seagrasses when inoculated on incubation chambers with *T. testudinum* with (a) epiphytes and (b) surfaced cleaned leaves.

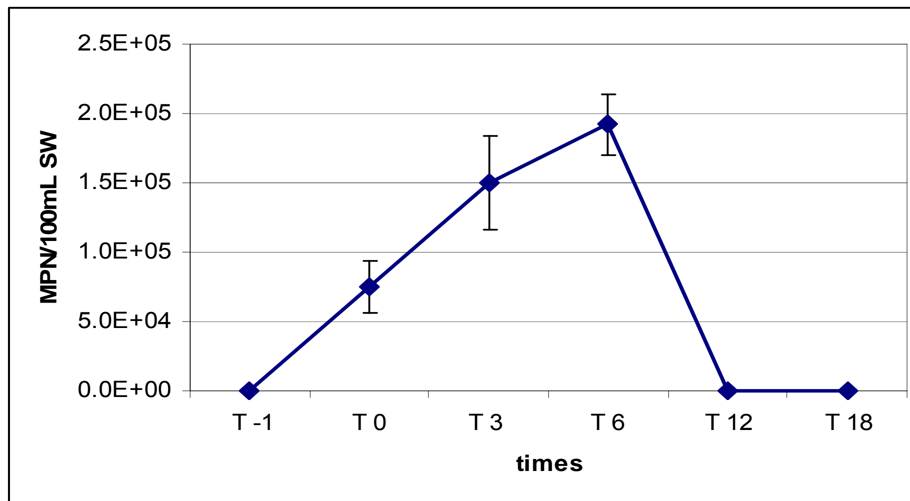


Figure 4.6. Dilution effect on the disappearance of *E. faecalis* (ATCC 29212) when directly inoculated on 37.5 L tanks. Water flow was suspended before T0 and re-initiated after T6 sample collection.

4.4 Discussion

In the present study, SW and SG enterococcal isolates from eight stations were analyzed over four different dates in terms of diversity and species composition as a preamble for MST. At least 22 enterococcal species were collected in La Parguera including FP and non-FP indicators and enterococcus-like bacteria (Figures 4.2 and 4.3). Enterococcal diversity varied for both SW and SG, and was mainly affected by closeness to the shore and by environmental factors such as precipitation and bird nesting (Table 4.1). The high species diversity that was evident during May and July was probably due to cattle egrets nesting in stations 3 and 4. A previous study suggests higher diversity of enterococci in environmental samples influenced by warm-blooded animals, in different European countries (Kühn *et al.*, 2002). During October, when rainy weather conditions prevailed, 18 species of enterococci were isolated, while during drier climate conditions (February) only four were identified, from which only two were enterococci used as FP indicators (i.e. *E. faecalis*, *E. faecium*, and *E. hirae*). Similar observations were described by Harwood, 2007, in which the abundance and diversity of enterococcal species was affected by environmental conditions with more diverse populations during events of high precipitation.

The species equitability among stations was also variable. Most of the stations presented high equitability, suggesting similar proportions of the species present for each sample, therefore, indicating no species dominance. Bird Island stations were one of the few exceptions, presenting low equitability at certain times. During May, for example, *E. gallinarum* was the dominant species in SW (33% of 4,821 CFU/100 mL and 54% of 6,234 CFU/100 mL at stations 3 and 4 respectively) followed by *E. faecium*

(27% and 19% for stations 3 and 4). These two species were also present in July, when enterococcal numbers were >600 at the same station, thus indicating a relation with cattle egret. The higher abundance of *Enterococcus gallinarum* makes it an indicator of fecal contamination by cattle egrets, in this case. However, during July, enterococcus-like species were approximately 50% of the isolates.

The enterococci suggested as human FP indicators were abundant and widely distributed. As previously suggested, *E. faecalis*, *E. faecium*, and *E. hirae* were used as indicators of human derived FP instead of the whole *Enterococcus* spp. assemblage (Bonilla *et al.*,2006 ; Kuntz *et al.*, 2004; Wheeler *et al.*, 2002). Nine of the 36 overtime sampled stations presented FP. Three of these stations indicated FP, but when performing the speciations, they revealed that the total FP indicator enterococci were bellow the U.S. EPA limit (Figure 4.2). This confirms the importance of performing enterococcal speciations to avoid overestimation during human derived FP events.

Enterococcus faecalis, the main human FP indicator, was not the dominant species of enterococci, not even in station 1 where human related pollution was expected because of the overflow of a manhole and increased runoff, especially during October. Niemi *et al.*, 1993, found also that when sampling for FP from various sources such as domestic and industrial wastewater, contaminated fresh and seawater, well water, cow dung, bird droppings, and pristine waters, *E. faecalis* was not the dominant enterococci among the enterococcal community. In the present study, *E. faecium* and *E. hirae* did occurred in station 1 during October, but their count was bellow the U.S. EPA

limit. Although the FP indicator enterococci were present all over sampled stations, these species did not show associations with specific sources.

Other warm-blooded related enterococci that are not typically indicators of human FP were also present and even dominant in certain conditions. As mentioned previously, *E. gallinarum* was the most abundant of the enterococci in SW samples examined in the current study, especially at stations 3 and 4 during the presence of cattle egrets. This enterococcal species has been described as an occasional component of the microbial flora of different animals including horses, cats, and dogs and rarely in chicken and humans (Aarestrup *et al.*, 2002). *Enterococcus villorum* was also present in high numbers in many of the stations including Bird Island, especially on SG (up to 11,576 out of 31,092 cells/g dry wt during May). This species have been previously associated to enteric disorders in pigs (De Graef *et al.*, 2003). In addition, *E. asini* was found in station 2 during cattle egret nesting (<30 CFU/ 100mL SW), a species that has been isolated from donkeys (De Vaux *et al.*, 1998). This opens the possibility of cattle egrets as zoonotic vectors together with the occurrence of pathogenic species in the area and so the introduction of antibiotic resistance genes into the environment initially associated to farming. Development of resistance to antibiotics, such as vancomycin, has been well described for enterococci associated with farm animals (Harwood *et al.*, 2000). This is a growing concern due to the possible dispersion of antibiotic resistance genes throughout the genus (Lester *et al.*, 2006). Antibiotic resistant enterococci were not studied during this investigation, but the possibility of the egrets being zoonotic vectors highly encourages further research on the presence/absence of antibiotic resistance genes in La Parguera.

Enterococcus-like bacteria were common in our samples. When pooling the data from all sampling times and stations, enterococcus-like bacteria were the dominant specie. In another study from La Parguera, significant differences between the frequency of violations of bacteriological standards and densities of enterococci were occasionally found offshore (Ramirez-Toro *et al.*, 2005) as well as in the present study when considering the whole *Enterococcus* spp. assemblage. When performing enterococcal speciations, the enterococcal counts on the SW samples in the off-shore sites were not necessarily associated to a human FP event. As it was previously mentioned, at least three areas, including Corral Reef and also Magueyes stations, presented FP, based on total enterococci, but no human fecal pollution was found when considering species composition (Figure 4.2). The presence of enterococci-like bacteria played a significant role in the overestimation of human derived FP.

Seagrasses presented a high number of enterococcus-like bacteria (up to a 60% of the total enterococci during October; Figure 4.3). In a Brazilian study, in which enterococci from mangrove oysters were analyzed, enterococcus-like bacteria accounted for 24% of the total enterococcal counts (Silva *et al.*, 2003). This indicates that enterococcus-like organisms can be found in different marine systems. *Thalassia testudinum* also showed to host previously described enterococci (Figure 4.3), including FP indicator enterococci, even when FP was not evident on the SW samples. For example, during October in sation 1, human FP indicator number was 46 CFU/100 mL for SW, while in SG sample, they accounted up to the 50% of 1,329 cells/g dry wt (Figures 4.2 and 4.3). Even though *T. testudinum* is able to host enterococci, it can not be concluded that SG is a primary reservoir and source of enterococci when high

enterococcal numbers are found in a sample, because as this study shows plant related enterococci (ie. *E. casseliflavus* and *E. mundtii*) were not found in SW. In addition, in samples where SG was affected by the constant movement of the shuttle boat (station 2), SG vs SW species composition, were not similar. The different species were not persistent over time and were not specifically found at any of the stations (Figure 4.3). This suggests enterococcal occurrence on SG but not persistence or long term association, suggesting that the incidence of enterococci is more related to environmental conditions that increases potential nutrient inputs, such as sediment runoff and sediment resuspension by boat traffic (stations 1 and 2) or by alternative fecal sources such as cattle egret.

Direct inoculation of *E. faecalis* onto seagrasses indicated that *T. testudinum* was not a good substrate or host for this species during this study. The present results indicate that marine environments do not support enterococci for longer than 12 h and that the dilution factor present after box removal at Corral Reef and water flow re-initiation on the *in vitro* study explains the drastic decrease of enterococci numbers. Several reports suggest that enterococci can replicate when released from its original source, e.g. warm blooded animals (Hartel *et al*., 2004, Whitman *et al*., 2005). Bordalo *et al*., 2002 suggest that enterococci might survive in seawater for 127 h in tropical regions. Enterococcal survival in the environment have been demonstrated to be inoculum-size dependent (Neely and Maley, 2000).

Rapid dilution of enterococci in SW may also explain the differences of SW and SG species composition in the environmental samples. During night time, currents

reduce speed sufficiently for dominant species of enterococci to settle down on the sediment. *Enterococcus faecalis* did not show attachment onto SG in the direct inoculation experiments, but without water flow the population increased (Figures 4.4 and 4.5). No attachment to the SG was evident in all assays, however, *E. faecalis* showed some adaptation to the environment of the epiphyte-controlled *T. testudinum*. In a study of bacterial and fungal biomass of the seagrass *Zostera marina* from Chesapeake Bay, fungi accounted for less than a 0.5% of the total community composition (Newell, 1981) suggesting that fungi is not a potential enterococcal inhibitor in submerged aquatic systems. It has also been previously reported that *T. testudinum* produces a chemical compound against zoosporic fungi and other fouling microorganisms (Jensen *et al.*, 1998), organisms that may inhibit enterococci growth. Other factors that may influence the lack of attachment of enterocci included: (i) inoculum size (Neely and Maley (2000), (ii) insufficient exposure time (6 h) for adaptation to the environmental conditions (iii) antimicrobial substances, and (iv) predators, pathogens, or competition with fouling organisms, as it was observed in the epiphyte-controlled assay. The direct inoculation study should be repeated taking in consideration new variables such as different inoculum size, other enterococcal species, other *E. faecalis* strains, including those isolated from SW and SG. Use of diffusion chambers might also add a new perspective (Bordalo *et al.*, 2002; Perez-Rosas and Hazen, 1988).

In conclusion, the results obtained in this study show a large temporal and spatial variation in terms of diversity in enterococcal abundance and community composition at

the study site. There was no specific pattern seen in the different stations in terms of species composition. *Enterococcus fecalis*, *E. faecium*, and *E. hirae* occurred in sites where no human derived FP was expected and also in different proportions. *Enterococcus faecalis* was not isolated where expected. Typically human-related enterococci were present in almost all of the stations in which enterococci were isolated, but in variable ranges and some times in low numbers where FP was expected (Figure 4.3). Although at low numbers, the human FP indicator species were found at other stations away from human fecal sources, suggesting that they can naturally occur in marine waters and related ecosystems.

Thalassia testudinum was found to be a host for enterococci especially during periods of “nutrients” input into the area, indicating this seagrass specie as a potential non point source of fecal indicator organisms, at least for short periods of time. These enterococci included warm and non-warm blooded-animals related enterococci as well as enterococcus-like bacteria.

The presence of enterococci, such as *E. gallinarum* indicate the presence fecal contamination and thus nutrient inputs derived from non-human warm-blooded animals. The use *E. gallinarum* - cattle egret relation is recommended in future studies for MST purpose. With the exception of the *E. gallinarum* /*E. faecium* to cattle egret relationship, the enterococcal species composition of our samples did not provide sufficient information for the identification of the source of human fecal inputs. The results provided some information that could have implications for beach and coastal testing for

FP, in terms of occurrence of enterococcus-like bacteria and other non warm-blooded related enterococci. Based on the low dominance of *E. faecalis* in areas prone to fecal contamination and its presence where no direct input of contamination was expected, an evaluation of the conventional methods for enterococcal detection is suggested.

CHAPTER 5: Conclusions

5.1 Conclusions

- This is the first study on enterococcal species composition for La Parguera that included an underwater marine plant and probably the first report on the occurrence of enterococci in *Thalassia testudinum*.
- *Enterococcus* spp. diversity was variable and was mainly affected by distance to shore and by specific events such as cattle egret nesting/roosting. Precipitation showed to be a possible factor influencing enterococcal input in La Parguera.
- *Thalassia testudinum* was able to host different enterococcal species, including the typically human related enterococci (*E. faecalis*, *E. faecium*, and *E. hirae*). Also, terrestrial plant associated enterococci, such as *E. casseliflavus* and *E. mundtii* were found at different sites and dates. In general, no particular species selection was observed at any of the sites.
- *E. faecalis* (ATCC 29212) did not show attachment to *T. testudinum*, when directly inoculated on this SG specie, during this study. The dispersion rate was the main cause of the rapid disappearance of enterococci. Still, this enterococcus attained a faster adaptation in the presence of epiphytes during controlled conditions.
- With exception of the relation between *E. gallinarum* and *E. faecium* with cattle egret colonies, no substantial evidence for the presence of human derived and other fecal pollution sources was found if speciation is included in the analysis. .
- A re-evaluation of the methods used for *Enterococcus* spp*.* isolation and consequently the standards used for our tropical coasts is suggested. The most

probable number approach with Enterococcosel Broth presented in this study is economically accessible, but still needs to be improved.

- Enterococcal abundance should be complemented with enterococcal speciation in order to determine if the enterococci present are an environmental contribution or actual fecal pollution indicators.

5.2 Future Work

- The use of enterococci as source trackers should be investigated in areas where higher human pollution impact is expected, such as nearby estuaries.
- Other techniques of MST are available and can bring faster results. These include other biological techniques as well as molecular, chemical, and bio-optical approaches.
- The viability and precision of using the catalase examination as a primary confirmation test when using Enterococcosel Broth as an isolation media should be tested as an alternative to save time in a situation that requires fast decisions and action for the proper control of a FP event. This also can function as an alternative for saving materials by confirming with BHIA 6.5% only the catalase negative bacteria instead of the total esculin positive bacteria.
- The direct inoculation study should be repeated taking in consideration new variables such as modifications in the water flux suspension, different inoculum size, and other enterococcal species and *E. faecalis* strains, including those isolated from SW and SG.

CHAPTER 6: References

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CHAPTER 7: Appendixes

Appendix 7.1. Enterococcus spp.: diversity of species and biochemical key

Group I: Argine negative, Sorbose and Mannitol positive

Organism	ARA	RIB	MGP	SUC	PIG	Source	ref
E. avium	+	+	+	+	-	Birds	4, 14
E. malodoratus	-	+	-	+	-	F, N	4, 14
E. raffinosus	+	+	+	+	-	ND	4, 14
E. pseudoavium	-	+	+	+	-	ND	4, 14
E. saccharolyticus	-	ND	+	+	-	ND	4, 14
E. pallens	+	+	-	+	+	C.I.	21
E. gilvus	-	+	-	+	+	C.I.	21
E. phoeniculicola	+	+	ND	+	+	Woodhoopoe (bird)	13
E. devriesei	v	+	-	+	-	D	18

Group II: Arginine and Mannitol positive, Sorbose negative

Organism	ARA	RIB	MGP	SUC	PIG	Source (isolated)	Ref
E. faecalis	-	+	-	v	-	H, some birds, D, N	1,3, 4, 14,23
E. faecium	+	+	-	v	-	H, D	4, 14
E. casseliflavus	+	+	+	+	+	P	4, 14
E. mundtii	+	+	-	+	+	P	4, 14
E. gallinarum	+	+	+	+	-	D	4, 14
E. seriolicida	-	-	-	v	-	Fish and eel / Japan	4, 14
E. flavescens	+	-	+	ND	+	C.I.	4, 14
E. solitarus	-	-	+	ND	-	ND	4, 14
E. haemoperoxidus	-	+	+	+	+	C.I.	16

Group III: Arginine positive and Sorbose and Mannitol negative

Organism	ARA	RIB	MGP	SUC	PIG	Source	Ref
E. durans	-	+	-	-	-	H, piglets	1,4,14,23
E. hirae	-	+	-	+	-	Chicken	1,4,10,14
E. dispar	-	+	+	+	-	H	4, 14
E. ratti	-	+	-	-	-	Rats with diarrhea	20
E. villorum	-	ND	-	-	-	Piglets with diarrhea	5,20
E. faecalis	-	+	-	-	-	D	4, 14
E. faecium	+	+	-	v	-	D	4, 14
E. canintestini	-	+	+	+	-	Dogs	15
E. silesiacus	-	+	-	-	-		19

Group IV: Arginine, Sorbose, and Mannitol negative

Organism	ARA	RIB	MGP	SUC	PIG	Source	Ref
E. asini	-	-	(-)	ND	-	Donkeys	6
E. sulfureus	-	+	+	+	+	ND	4, 14
E. cecorum *	-	+	-	+	-	Pigeons, D	1,4,14
E. aquimarinus	+	-	-	+	-	SW	17
E. termitis	-	+	+	-	-		19

Group V: Arginine and Sorbose negative, Mannitol positive

Organism	ARA	RIB	MGP	SUC	PIG	Source	Ref
E. columbae *	+	+	d	+	-	pigeons	1,2,7,14
E. canis	+	+	+	(-)	-	Dogs	5
E. moraviensis	+	+	+	+	-	C.I. , W	16
E. faecalis	-	+	ND	ND	-	D, H	1
E. casseliflavus	+	+	ND	ND	+	P	1
E. gallinarum	+	+	ND	ND	-	D	1
E. hermanniensis	-	+	ND	-	?	F, dogs	12
E. italicus	-	-	+	+	-	F	11

Two species from the *Tetragenococcus* genus. This genus is similar and can be confused with *Enterococcus spp*

Organism	ARG	ARA	SOR	MAN	RIB	MGP	SUC	PIG	Ref
T. halophilus	ND	+	-	-	+	-	+	ND	9
T. muriaticus	ND	-	-	+	+	-	-	ND	9

ARG=arginine; ARA=arabinose; SOR=sorbose; MAN=Mannitol; RIB=Ribose; MGP= α-D-Methylglucoside; SUC= Sucrose; PIG= pigmentation. Source= main source or where it was found, d= different data on references; + = more than 90%; - = less than 10% ; (+)= 75-89% positive ; (-)= 11-24% positive, v=variable (25-74% positive); ND= No data. * *E. cecorum* y *E. columbae* are susceptible to 0.4% Sodium Azide (enterococcocel). Sources: F=foof, N= naturally occurring, C.I. = clinical isolate, D=different sources, H= human, P= plant associated, W= water, SW= seawater

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http://www.bacterio.cict.fr/e/enterococcus.html

http://www.bacterio.cict.fr/bacdico/ee/enterococcus.html

Appendix 7.2 Procedure for biochemical examination

***Courtesy of Peter Hartel, Ph.D. Department of Crop & Soil Sciences, University of Georgia**

Recipes:

Phosphate buffer

To 1 liter of distilled water add 8.5g of NaCl, 0.65g of K_2HPO_4, and 0.35g of KH_2PO_4. Filter-sterilize or autoclave.

Arginine decarboxylase medium:

This can be made the day before speciation.

Arginine negative:

To 100mL of distilled water add 0.90g of decarboxilase medium and 0.25g agar. Autoclave. If made the day before speciation store at room temperature.

Arginine positive:

To 100mL of distilled water add 0.90g of decarboxilase medium, 0.25g of agar, 0.5g of L-arginine. Autoclave. If made the day before speciation store at room temperature.

Agar and dye

To 100mL of distilled water, add 2.5g agar and 0.04g of bromthymol blue. Autoclave and then place in a water bath set at 50°C. Do not allow agar to solidify.

Mineral Medium

The day of the speciation combine 450mL of distilled water with 3.30g of NaCl, 0.66g of KH_2PO_4, 0.66g of NH_4NO_3, 0.132g of MgSO4, and 0.132g of yeast.Adjust pH to 7.4 with 1 M NaOH. Divide the solution into 90mL aliquots. To each aliquot, add 1.1g of a carbohydrate (arabinose, sorbose, manitol, methyl α-D-glucopyranoside, ribose, or sucrose). Check pH of each and adjust to 7.4 with M NaOH. Filter-sterilize each through a 0.45 or 0.22μm filter and place into a 50°C water bath.

Procedure:

Two days before speciation:

Inoculate one pure colony of confirmed enterococci into 96-well plates containing 150μL Enterococcosel Broth per well. Use one well for each bacteria to be speciated, leaving 8 wells uninoculated for 5 known enterococci (ie. *E. faecalis*, *E. faecium*, *E. gallinarum*, among other) to be used as controls, at least one non enterococcal bacteria (ie. *E.coli*, *Staphylococcus* spp.,etc), and two blanks. Incubate at 37°C. Number of plates to be used will depend on the number of isolates.

Day before the speciation:

Prepare saline phosphate buffer, argine test (if desired) and agar and dye.

Day of speciation:

Serve 125µL of saline-phosphate buffer aseptically to each well of the microtiter plates with a multichannel pipetter. Number of plates will depend on the number of enterococci previously inoculated on enterococcosel for identification purpose. Prepare carbohydrate mineral medium. Add 10 mL of the dye plus agar to each mineral medium. Transfer 150µL of each medium (argine positive and negative and carbohydrates) to each well of a microtiter plate using a multi-channel pipette. Transfer 10µL from the enterococci plate to the buffer plate using a sterile replicator. Make sure to label each buffer plate in concordance with the correspondent enterococcosel plate as well as each of the plates containing the media. Place a sterile replicator into one of these plates and transfer the bacteria to each corresponding plate with media. Use a fresh replicator for each plate. Add 75 µL of sterile mineral oil to the top of each medium. Incubate at 37°C for 72 hours.

Results reading:

Arginine negative plates: a yellow well is recorded as positive, if purple is recorded as negative. Any negative well is automatically recorded as negative in the arginine positive plate.

Arginine positive plates: for the remaining wells (the ones that were recorded as positive on the arginine negative plates), purple wells are considered positive and yellow ones negative.

Carbohydrates: A well is recorded as positive if is yellow or green and negative if it remains blue.

Appendix 7.3. Analysis of variations on enterococcal isolation media and confirmation procedure.

Isolation Methods:

Col 2 = enterococcosel
Col 3= BHIA 6.5%NaCl
Col 4 = catalase test

1 variable: MPN/100mL . Decay during confirmation tests

One Way Analysis of Variance

Data source: Data 2 in Notebook 1

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 2 in Notebook 1

Group	N	Missing	Median	25%	75%
Col 2	112	0	88.269	28.558	326.499
Col 3	112	0	28.558	0.000	211.755
Col 4	112	0	28.558	0.000	157.849

H = 13.918 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
Col 2 vs Col 4	5213.000	5.071	Yes
Col 2 vs Col 3	3478.000	3.383	Yes
Col 3 vs Col 4	**1735.000**	**1.688**	**No**

this result indicates that confirmation tests must be performed at least until 6.5% NaCl

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Appendix 7.4. Spatial and temporal composition of Enterococcus spp. from seawater and T. testudinum samples.

*** M.I: Magueyes Island, B.I.: Bird Island, E.R.: Enrique Reef, C.R.: Corral Reef**

SW: seawater, SG: seagrasses

May 19, 2005	M.I. 1		M.I. 2		B.I. 1		B.I 2		E.R. 1		E.R. 2		C.R. 1		C.R 2	
	SW	SG	SW	SG	SW	SG	SW	SG	SW	SG	SW	SG	SW	SG	SW	SG
E. faecalis II	0	0	0	217	0	3035	779	0	0	0	0	0	0	0	0	0
E. faecium II	0	58	30	786	1286	0	1169	5390	0	0	0	0	0	0	0	0
E. casseliflavus	0	0	0	0	0	1517	0	0	0	0	0	0	0	0	0	0
E. mundtii	0	0	0	0	0	3035	0	0	0	0	0	0	0	0	0	0
E. gallinarum II	29	0	0	217	1607	0	3377	0	0	0	0	0	0	0	0	0
E. seriolicida	0	58	0	217	321	1059	130	13	0	0	0	0	0	0	0	0
E. solitarus	0	0	0	108	107	353	0	0	0	0	0	0	0	0	0	0
E. haemoperoxidus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*GIII	0	58	30	0	0	0	130	0	0	0	0	0	0	0	0	0
**GIII	0	0	0	0	0	1517	0	2637	0	0	0	0	0	0	0	0
E. hirae	0	87	15	1098	0	0	130	1331	0	0	0	372	0	0	0	0
E.villorum	0	204	45	150	750	11576	0	5274	0	0	0	0	0	0	0	0
E. faecium III	0	0	0	217	107	353	130	0	0	0	0	0	0	0	0	0
E. asini	0	29	0	217	0	0	0	0	0	0	0	0	0	0	0	0
E. cecorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***GV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. columbae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. canis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. hermanniensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ent-like	0	117	15	231	643	8646	390	3994	0	0	0	124	0	0	0	0

July 8, 2005	M.I. 1		M.I. 2		B.I. 1		B.I 2		E.R. 1		E.R. 2		C.R. 1		C.R 2	
	SW	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**
E. faecalis II	6	107	0	0	0	2275	22	8194	0	0	0	0	0	0	0	0
E. faecium II	6	0	0	68	41	1609	36	5400	0	0	0	0	0	0	0	0
E. casseliflavus	0	0	0	0	0	569	0	0	0	0	0	0	0	0	0	0
E. mundtii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum II	0	0	0	135	81	15161	86	9841	0	0	0	0	0	0	0	0
E. seriolicida	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0
E. solitarus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. haemoperoxidus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*GIII	0	0	0	0	51	0	14	0	0	0	0	0	0	0	0	0
**GIII	0	0	0	0	20	0	22	1772	0	0	0	0	0	0	0	0
E. hirae	0	200	0	338	61	2698	65	12656	0	0	0	0	0	0	0	0
E.villorum	0	0	0	0	31	0	7	0	0	0	0	0	0	0	0	0
E. faecium III	0	133	8	100	153	5893	65	14491	0	0	0	0	0	0	0	0
E. asini	0	0	0	0	31	1065	7	3649	0	0	0	0	0	0	0	0
E. cecorum	0	0	0	0	20	520	7	897	0	0	0	0	0	0	0	0
***GV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. columbae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. canis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. hermanniensis	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0
ent-like	9	54	0	405	387	14435	302	16575	0	0	0	0	0	0	0	0

October 14, 2005	M.I. 1		M.I. 2		B.I. 1		B.I 2		E.R. 1		E.R. 2		C.R. 1		C.R 2	
	SW	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**
E. faecalis II	0	131	0	216	0	171	0	0	0	0	14	0	0	0	0	0
E. faecium II	15	398	51	2068	0	343	0	758	0	0	0	0	52	0	102	0
E. casseliflavus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. mundtii	0	131	0	0	0	189	0	0	0	0	0	0	0	0	0	0
E. gallinarum II	31	8	0	3891	0	257	0	189	0	0	0	0	224	0	102	0
E. seriolicida	0	0	0	216	0	0	0	0	0	0	0	0	0	0	0	0
E. solitarus	0	0	0	216	0	0	0	0	0	0	0	0	0	98	0	213
E. haemoperoxidus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*GIII	15	4	0	648	0	0	14	0	0	0	0	0	0	0	0	0
**GIII	0	131	0	216	0	0	0	0	0	0	0	0	0	0	0	0
E. hirae	0	0	0	648	15	0	0	0	0	0	0	0	0	0	0	0
E.villorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0
E. faecium III	0	131	0	0	30	0	0	0	0	0	0	0	0	0	0	426
E. asini	0	0	0	0	0	0	0	379	0	0	0	0	0	98	0	0
E. cecorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***GV	15	0	17	0	0	0	0	0	0	0	0	0	0	197	0	0
E. columbae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	213
E. canis	0	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis V	0	0	0	0	0	0	0	0	0	0	14	0	0	98	0	0
E. gallinarum V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	1
E. hermanniensis	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0
ent-like	31	394	51	2923	45	2	0	379	0	0	0	0	103	500	68	1278

February 13, 2006	M.I. 1		M.I. 2		B.I. 1		B.I 2		E.R. 1		E.R. 2		C.R. 1		C.R 2	
	SW	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**	**SW**	**SG**
E. faecalis II	0	0	0	0	0	0	0	151	0	0	0	0	0	0	0	0
E. faecium II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. casseliflavus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. mundtii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. seriolicida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. solitarus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. haemoperoxidus	0	0	0	0	0	0	29	132	0	0	0	0	0	0	0	0
*GIII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
**GIII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. hirae	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0
E.villorum	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0
E. faecium III	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. asini	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. cecorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
***GV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. columbae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. canis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. hermanniensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ent-like	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0