USE OF THE *ESP* GENE TO DETECT HUMAN CONTAMINATION WITH *ENTEROCOCCUS FAECIUM* OF SOUTH TEXAS COASTAL WATERS

by

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May 2016

A Thesis Submitted
In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE IN BIOLOGY

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Format: *Journal of Applied Microbiology*

ABSTRACT

Microbial source tracking comprises a set of techniques to determine animal/human sources of fecal contamination. As new methods are developed, it is important to evaluate their effectiveness in a range of environments. The enterococcal surface protein gene (*esp*) of *Enterococcus faecium*, which has been used to indicate the presence of this human-associated opportunistic pathogen, was used in this study to detect human-source contamination in marine and freshwaters of south Texas. Several surface water segments in this area have been placed on the Texas 303 (d) list of impaired waters for bacteria contamination, as a result of elevated levels of enterococci or *Escherichia coli*. Detection of *esp* was compared with levels of fecal indicator bacteria in the water in order to determine whether contamination was of human origin and also to assess whether the contamination was detected at times of elevated indicator bacteria levels. Water samples were collected from marine and freshwater environments in the Texas Coastal Bend area, and fecal bacteria levels were determined using standard membrane filtration methods. For *esp* analysis, water was filtered onto membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) and incubated at 41°C for 48 hours. Following enrichment in azide dextrose broth, DNA extraction and PCR analysis were performed. The *esp* gene was detected in both marine and freshwater samples by PCR. Inhibition frequently affected PCR analyses in initial samples from marine sites, so a protocol for inhibitory samples was used for the remainder of the study. The *esp* gene was only detected in marine waters when *Enterococcus l*evels greatly exceeded water quality standards. The lowest enterococci concentration of an *esp*-positive freshwater sample was 83 CFU 100 ml^{-1}. The *esp* gene

showed potential for use in detecting human source contamination in Texas coastal waters.

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ACKNOWLEDGMENTS

The study was funded by the Texas GLO, Coastal Bend Bays and Estuaries Program (Ropes and Cole parks), and TSSWCB (Oso Creek). Thank you to my graduate advisory committee members, Dr. Joanna Mott, Dr. Gregory Buck, and Dr. Patrick Larkin, for their guidance and flexibility. Thank you to Roger Sealy for being my partner in Ropes and Cole parks sampling and to Marc Carpenter for collecting all the freshwater samples from the Oso Creek TCEQ sites. Thank you to my fellow graduate students, Summer Shi, Sergio Rodriguez, and Miguel Diaz, and to all the member of the Environmental Microbiology Lab for their help and support. Thank you to my husband, Kyle Lindsey, for his love and encouragement in finishing this project.

INTRODUCTION

Background

Corpus Christi Bay is located in the Coastal Bend area of south Texas. The surrounding semi-arid land is used for ranching and other agricultural purposes, and the Corpus Christi Metropolitan Statistical Area (Aransas, Kleberg, Nueces, and San Patricio counties) supports a human population of 460,400 as recorded in the 2010 United States Census (United States Census 2010). Corpus Christi Bay extends west to Nueces Bay, fed by Nueces River, and south to Oso Bay, fed by Oso Creek. According to the Corpus Christi Convention and Visitors Bureau, the tourism industry in the Coastal Bend supports around 13,000 jobs and brings 1.1 billion dollars into the local economy annually, with nature and wildlife activities such as beach strolling, bird watching and fishing accounting for 40% of all visitor trips (Coastal Bend Bays and Estuaries Program 2010). In an effort to enhance cooperation between businesses, government, and the general public in the management of the bay and estuaries, the United States Environmental Protection Agency (USEPA) designated the Corpus Christi Bay system as an estuary of national significance with the creation of the Corpus Christi Bay National Estuary Program (USEPA 1999).

Estuaries provide many benefits to humans: agricultural support, water supply, commercial and sport fisheries, recreational uses such as swimming and boating, and discharge points for municipalities and industries. Bay waters may become polluted when rainwater washes pollutants from yards, streets, farms, construction sites, or poorly performing sewage treatment plants and septic tanks. These pollutants have the potential

for causing acute and chronic health effects in humans, either by direct contact through activities such as swimming, or by eating seafood that has been exposed to harmful pollutants or pathogens (USEPA 1999). For example, gastrointestinal, respiratory, ear/nose/throat, eye, skin and mucosal symptoms in swimmers at polluted beaches have been reported in several recreational water studies (Cabelli *et al.* 1979; Pruss 1998; Marion *et al.* 2010). Fecal bacteria from humans and animals pose a health risk to humans, with the most common result of exposure to fecal contaminated water being gastroenteritis (Kay *et al.* 1994; USEPA 2009).

Fecal Bacteria as Indicators for Risk to Human Health

Fecal indicator bacteria densities are used to measure the sanitary quality of recreational water (USEPA 2000; USEPA 2009). Use of indicator bacteria precludes the need for individual testing of each type of pathogen associated with fecal contamination (Scott *et al.* 2002; USEPA 2009). Ideal indicators are nonpathogenic, easily detected and enumerated, have similar survivability to fecal pathogens, and are strongly associated with the presence of fecal pathogens (Scott *et al*. 2002; Noble *et al*. 2003). Increasing levels of an ideal indicator in a body of water would be associated with an increased risk of illness (Wade *et al.* 2003). Enterococci and fecal coliforms, including *Escherichia coli,* have been used as fecal indicators because these bacteria are abundant in the feces of warm-blooded animals, including humans, and levels of these bacteria have been shown to correlate with illness of swimmers (Pruss 1998; Haile *et al*. 1999; USEPA 2000; USEPA 2009). Haile *et al.* (1999) found increased incidence of both upper respiratory and gastrointestinal symptoms, including vomiting, diarrhea, and sore throats, for subjects swimming in ocean water subject to urban runoff. Elevated levels of fecal

indicator bacteria were observed near flowing storm drains, and an increased incidence of illness was observed in swimmers who had swum near the storm drains (Haile *et al.* 1999).

Enterococci are gram-positive, spherical or ovoid cocci that occur singly, in pairs, or in chains (Facklam *et al.* 2002). They are aerotolerant and possess a growth range of 10 to 45°C with an optimum growth temperature of 35°C (Facklam *et al.* 2002). *Enterococcus* species are separated into five groups based on phenotypic characteristics rather than phylogenetic relationships (Facklam *et al.* 2002). *Enterococcus faecalis* and *Enterococcus faecium* are the species most commonly found in humans (Scott *et al.* 2002). Enterococci are particularly useful as indicators in marine environments (Cabelli *et al.* 1982; Wade *et al.* 2003). They have been shown to mimic the survival of pathogenic organisms and correlate with gastrointestinal illness (Dufour 1984; USEPA 2000). However, a concern is that regrowth of these organisms is possible once they have been introduced into the environment, thus potentially compromising the indication of fecal contamination (Desmarais e*t al.* 2002). Environmental reservoirs of indicator bacteria result in indicator bacterial levels that are not a reflection of recent fecal contamination, resulting in unnecessary beach closures.

Microbial Source Tracking

A study in Mission Bay, California found no relationship between indicator organisms and illness rates, citing the low level of fecal contamination from human sources in Mission Bay as a possible reason for the lack of a relationship (Colford *et al.* 2007). However, a 2010 study using quantitative microbial risk assessment methods (QMRA)

concluded that recreational waters impacted by fresh cattle sources and human sources present a similar risk for gastrointestinal illness while exposure to waters impacted by fresh gull, chicken, or pig sources present a lower risk (Soller *et al.* 2010). Thus, effective management of contaminated surface waters and the evaluation of risk to human health require identification of the dominant source of fecal contamination (NRC 2004; Colford *et al.* 2007; Soller *et al.* 2010). A group of methods has been developed for microbial source tracking (MST) to identify sources of fecal contamination using indicator bacteria (Simpson *et al.* 2002). However, no single MST method developed to-date can identify all pollution sources with 100% accuracy.

MST methods can be divided into two groups: library-dependent and library-independent. Library-dependent methods identify bacteria from environmental samples by comparison to a library of known bacteria; library-independent methods do not require a library because they use molecular techniques to determine the presence of a known indicator or animal-specific organism instead of attempting to identify all bacteria present in the sample. Antibiotic resistance analysis (ARA), a library-dependent MST method, is a phenotypic characterization method that has been used in several Texas studies to identify fecal sources (Jones *et al.* 2007; Mott *et al.* 2008). Drawbacks to this library-dependent method include the necessity and expense of an extensive library database, changes in resistance, and regional specificity (Harwood *et al*. 2000). Because identifications are based on bacterial response to a set of antibiotics, a change in resistance or a difference of resistance between library bacteria and sample bacteria can cause an erroneous identification. Carbon source utilization (CSU) is another phenotypic characterization method that is based on comparison to a library. Bacteria of unknown

origin are classified by similarity in their utilization of a group of carbon sources (Jones *et al.* 2007). Library development is one of the most costly components of MST methods.

Library-independent methods rely on the detection of host-specific molecular markers, and results are qualitative if conventional Polymerase Chain Reaction (PCR) is used or semi-quantitative if quantitative PCR (qPCR) is used (Noble *et al.* 2006; Ahmed *et al.* 2007). A wide range of molecular markers from host-associated bacteria and viruses have been cited for use in library-independent MST. Host-specific targets within the *Bacteroides* 16S rRNA gene, present in feces of humans and animals, have been used to identify possible sources and levels of fecal pollution in watersheds and recreational waters in several studies (Bernhard and Field 2000; Gourmelon *et al.* 2007; Shanks *et al.* 2010). *Bacteroides* species are gram-negative, non-motile, anaerobic rod-shaped bacteria found in the gastrointestinal tracts of humans and animals (Smith *et al.* 2006). They have been suggested as an alternative fecal indicator bacteria because they are abundant in the fecal bacteria population, do not proliferate in the environment, have a similar survivability to pathogens, and are host-specific (Field and Dick 2004; Layton *et al.* 2006). The human polyomaviruses (HPyVs) have also been useful in library-independent MST. The BK virus and JC virus of the Polyomavirus family infect humans and are found in human populations worldwide (Eash *et al.* 2006). A 2012 study in Florida evaluated the HF183 marker for human-associated *Bacteroides* and the marker for human polyomaviruses using qPCR to determine specificity and limits of detection (Staley *et al.* 2012a). The HPyV assay produced zero amplifications in 32 non-target fecal composite samples, showing a specificity of 100%. The *Bacteroides* assay had an overall specificity

of 81.25%, showing cross-reactivity with dog, chicken, and duck fecal samples (Staley *et al.* 2012a). Library-independent methods are cost-effective and more rapid than library-dependent methods (Casarez *et al*. 2007). However, there are concerns regarding geographical stability of markers and the interpretation of results in relation to accepted regulatory water quality standards and human health (Casarez *et al.* 2007). Also, markers are not present in the feces of all individuals and may vary in concentration, so the absence of a human-specific marker does not eliminate the possibility of human fecal contamination (Field and Samadpour 2007).

esp Gene as a Library-independent Method to Detect Human Fecal Contamination

Enterococcal surface protein (Esp) is a high molecular weight surface protein found in species of enterococci associated with the human intestinal tract (Toledo-Arana *et al.* 2001; Scott *et al.* 2005; McQuaig *et al.* 2006). The protein is involved in biofilm formation by *E. faecalis* and *E. faecium* and is a virulence factor of human-associated *E. faecium* (Toledo-Arana *et al.* 2001; Leavis *et al*. 2004; Heikens *et al.* 2007). The *esp* gene has also been recently described as transferrable by conjugation as part of a pathogenicity island (Oancea *et al.* 2004; Top *et al.* 2011). Esp of *E. faecalis* shares up to 90% homology with Esp of *E. faecium,* and primers have been developed to target the *esp* gene of human-associated *E. faecium* (Scott *et al.* 2005; Heikens *et al*. 2007). When these human-specific primers are used, PCR-based detection of the *esp* gene indicates the presence of human-associated *E. faecium.* The *esp* gene has been used as a library-independent molecular MST method in several studies (Scott *et al.* 2005; McDonald *et al*. 2006; Brownell *et al.* 2007; Ahmed *et al*. 2008; Korajkic *et al.* 2009; Abdelzaher *et al.* 2010).

A study by Scott *et al.* (2005) examined the use of the *esp* gene as an indicator of human fecal contamination using sewage and wastewater samples from Florida, Arizona, and Michigan. To target the human-specific *esp* gene variant during PCR amplification, Scott *et al.* used a previously developed reverse primer (Hammerum and Jensen 2002) and designed a new forward primer specific to the *esp* gene found in human-specific *E. faecium*. They analyzed 65 human fecal samples and 102 animal fecal samples for the presence of *esp*. The human fecal samples included 40 samples from primary sewage influent, 10 samples from secondary sewage effluent, five samples from filtered wastewater, and 10 samples from septic tanks. The animal fecal samples included water samples from wastewater lagoons at swine, poultry, and dairy farms and individual fecal samples from Canada geese, poultry, swine, dairy cattle, beef cattle, seagulls, and pelicans. The *esp* gene was detected in 63 of the 65 human samples; *esp* was not detected in two of the eight septic tank samples. The authors note that these two septic tank samples had *Enterococcus* concentrations lower than 100 colony forming units (CFU). The *esp* gene was not detected in any of the 102 animal fecal samples. Thus, the overall sensitivity of the marker was 97%, and the specificity was 100%. Sensitivity is the probability of detection when the source is present, and specificity is the probability of no detection when the source is not present (Ahmed *et al.* 2009). Scott *et al.* also examined the persistence of the *esp* marker by inoculating freshwater and simulated seawater with raw sewage and analyzing for *esp* at days zero, three, five, seven, and 10 of a 30°C incubation. The *esp* gene was detectable up to day five in freshwater (total enterococci $1.22 \pm 0.30 \times 10^2$ CFU) and day 10 in simulated seawater (total enterococci $7.1 \pm 0.3 \times 10^1$ CFU) (Scott *et al.* 2005).

The *esp* marker was also used in a study of a west coast Florida beach to examine stormwater impact on water quality (Brownell *et al.* 2007). Using the same methods described by Scott *et al.* (2005) for *esp* detection, the authors analyzed water and sediment samples taken after a heavy rainfall and after a dry period, defined by six days of no precipitation. The *esp* gene was not detected in any of the samples, and the authors concluded that while human fecal contamination was not indicated, high numbers of fecal indicator bacteria and an increase in the *Enterococcus* population diversity during the rain event call for a better understanding of the persistence and ecology of indicator bacteria (Brownell *et al.* 2007).

A study by Staley *et al.* (2012b) measured the presence of *E. faecium*, *Bacteroides* species, and HPyVs in a Florida lake; *esp* was included as the indicator marker for enterococci. Of the MST markers, the *esp* gene demonstrated the only correlation between the presence of any marker and fecal coliforms (r^2=0.469, P<0.0001) and *E. coli* (r^2=0.194, P=0.013).

A study by Ahmed *et al.* (2008) supported the use of *esp* as a MST marker, citing 90.5% sensitivity and 100% specificity; the authors detected *esp* in 30 of 30 sewage samples and in eight of 12 septic tank samples but did not detect *esp* in 155 animal samples. In a similar 2009 study, human-specific *esp* was shown to be 100% specific and 100% sensitive using 32 sewage treatment plant samples and 50 animal fecal samples from cattle, pigs, sheep, dogs and ducks (Ahmed *et al.* 2009). In this study, real-time PCR was used to determine the minimum detection limit of various human-specific molecular markers. Sewage samples were suspended in freshwater, seawater and

distilled water in a 1:1 ratio (62.5 ml sewage: 62.5 ml water) and then diluted from 10^{-1} to 10^{-10}. The human-specific *esp* marker was detected in freshwater spiked with sewage up to a dilution of 10^{-4} with an enterococci concentration of $8.1 \pm 6.0 \times 10^{1}$ CFUs. The human-specific *esp* marker was detected in sewage spiked seawater up to a dilution of 10^{-4} with an enterococci concentration of $7.1 \pm 9.0 \times 10^{1}$ CFUs.

While some studies have found the *E. faecium esp* marker to be 100% human-specific (Scott *et al.* 2005; Ahmed *et al*. 2008; Ahmed *et al*. 2009), other research shows that the gene is present in non-human sources (Whitman *et al.* 2007). Though located on the bacterial chromosome (Oancea *et al.* 2004; Scott *et al.* 2005), the *esp* gene is transferable among enterococcal isolates via conjugation (Oancea *et al.* 2004). Humans live in close contact with domestic animals, making the horizontal transfer of genes between human and animal fecal bacteria, such as *Enterococcus*, likely (Dick *et al.* 2005). Whitman *et al.* (2007) detected the *E. faecium esp* marker in 4.7% of 233 animal fecal samples. Nine of 43 dog fecal samples and two of 34 gull fecal samples tested positive for the *E. faecium esp* marker, which had been presumed human-specific. The *esp* gene was not detected in goose, deer, cat, or raccoon fecal samples, and only the non-specific *esp* marker, which targets *E. faecalis* and *E. faecium,* was detected in songbirds and mice.

Monitoring Surface Water Quality in South Texas Using the *esp* Gene

In south Texas, surface water quality is monitored by several state agencies including the Texas Commission on Environmental Quality (TCEQ), Texas State Soil and Water Conservation Board (TSSWCB), and the Texas General Land Office (GLO). The Beaches Environmental Assessment and Coastal Health Act of 2000 requires that states

monitor for pathogens and pathogen indicators in coastal recreation waters at public bathing beaches and notify the public when standard limits are exceeded (Public Law 2000). Texas water bodies that do not meet the criteria defined in the Texas Surface Water Quality Standards are placed on the Texas 303(d) list in compliance with the Federal Clean Water Act Sections 305(b) and 303(d) (Public Law 1977; TCEQ 2010a). The Texas Beach Watch (TBW) Program, a non-regulatory program implemented by the Texas GLO, monitors enterococci levels at coastal beaches. When *Enterococcus* levels exceed limits established by the USEPA (single sample limits of 104 colony forming units per 100 ml [CFU 100 ml^{-1}] in marine or ocean water from the designated beach areas), the TBW Program issues an advisory for the beach or area and a sign is posted near the area (USEPA 1986; Texas General Land Office 2010). Two recreational parks with stormwater outfalls near residential areas along Corpus Christi Bay, Ropes and Cole parks, are monitored at six sites through the TBW Program. Both have a history of water exceeding the EPA recommended enterococci concentration limit of 104 CFU 100 ml^{-1}. A 2001 study by Mott *et al.* examined fecal indicator bacteria levels at Ropes and Cole parks over a seventeen month period that included six rainfall events. Water samples were analyzed for concentrations of enterococci, total coliforms, and fecal coliforms, and significant increases in fecal indicator bacteria levels were observed after rainfall, peaking within two days of rainfall. These parks have recently been included on the 2010 Texas 303(d) list as impaired for the parameter 'bacteria' (TCEQ 2010b).

Oso Creek (Segment 2485A), which flows into Oso Bay and ultimately Corpus Christi Bay, has been included on the Texas 303(d) list as impaired due to excessive bacteria since 2002 (Mott and Hay 2010; TCEQ 2010b). The creek receives effluent

from Robstown Wastewater Treatment Facility at levels permitted by the TCEQ and runoff from both urban and agricultural land. The TSSWCB funded a three year project that focused on determining nonpoint sources of enterococci in the upper sections of the creek and identifying which animals are contributing to loading in the Oso Creek watershed. The first year of the project included sampling of subsurface waters under dry and wet conditions, sampling at historic TCEQ stations and sampling of sediments and soil to identify nonpoint sources of fecal bacteria. The second year of the project included continued sampling and a MST study utilizing library-dependent CSU and ARA methods to identify animal (livestock and wildlife) and human sources of contamination.

In this study, detection of the *esp* gene was used as a MST technique to determine if human fecal contamination has been contributing to the bacteria loading at Ropes and Cole parks (marine waters) and the upper Oso Creek (freshwater).

Objectives

- To determine whether the *esp* gene can be used as an MST method for south Texas waters
- To determine whether there is a human source contribution to the contamination of Corpus Christi Bay (Ropes and Cole parks) marine waters, under both dry conditions and following rainfall, using the *esp* gene as an indicator of human fecal contamination
- To determine whether there is a human source contribution to the contamination of the upper Oso Creek, using the *esp* gene as an indicator of human fecal contamination

MATERIALS AND METHODS

Study Area

This project involved the collection of marine and freshwater samples from south Texas coastal waters near the city of Corpus Christi, Texas. Marine water from Corpus Christi Bay was collected from two sampling sites at Ropes Park and four sampling sites at Cole Park. Freshwater samples were collected from three historic TCEQ water quality monitoring stations located on Oso Creek and West Oso Creek, one station located downstream of Robstown Waste Water Treatment Facility, and one station located immediately upstream of USGS gauge #08211520 as described in a Quality Assurance Project Plan (QAPP) for the Texas State Soil and Water Conservation Board (Mott and Hay 2010). A map of the study area and sampling locations is shown in Figure 1. Figure 2 is a satellite image of Ropes Park with NUE028 and NUE029 marked in green. Figure 3 is a satellite image of Cole Park with NUE031, NUE032, NUE033, and NUE035 marked in yellow. GPS coordinates for the sampling locations are listed in Table 1.

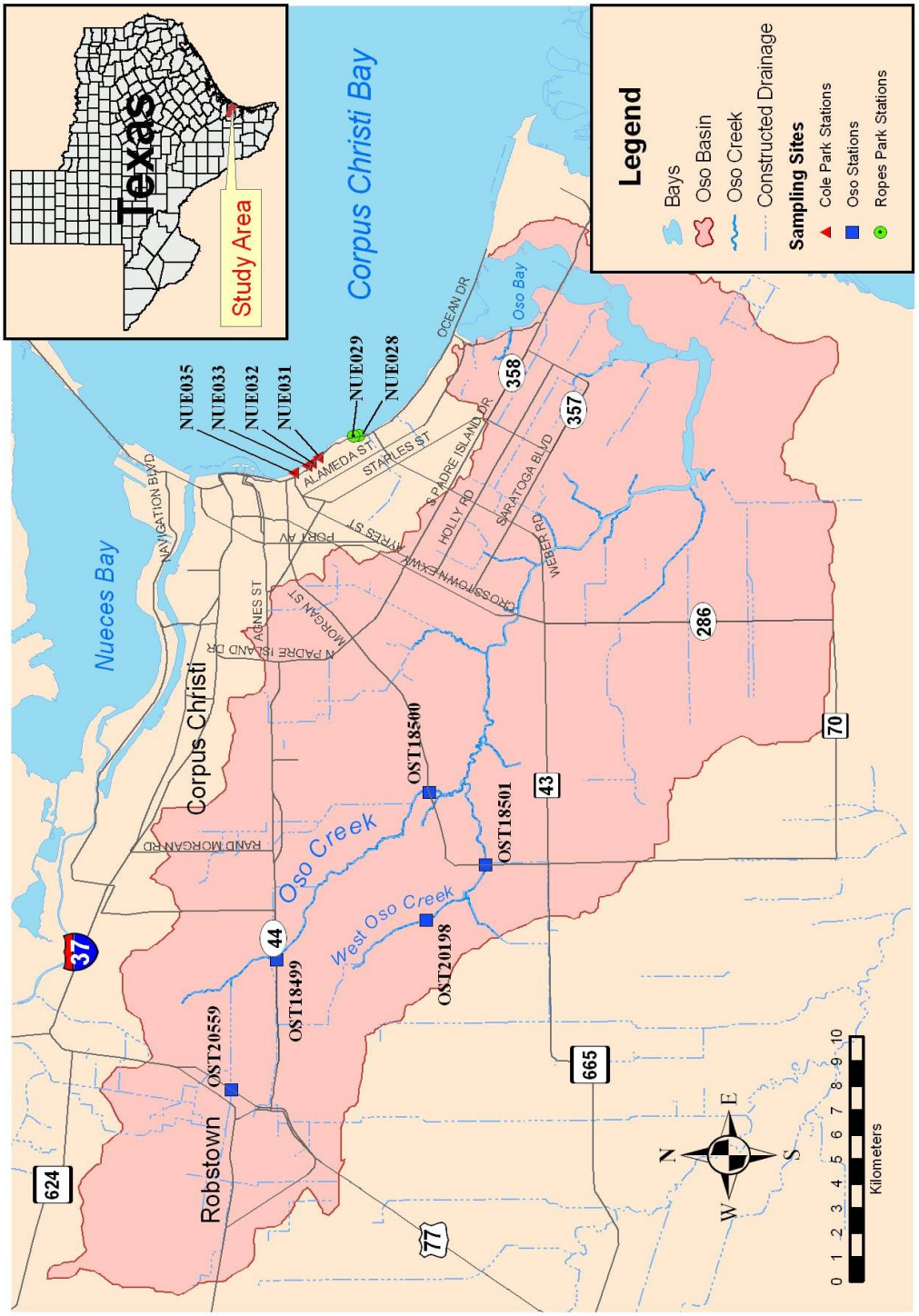

Fig. 1 Map showing study area with sampling locations.

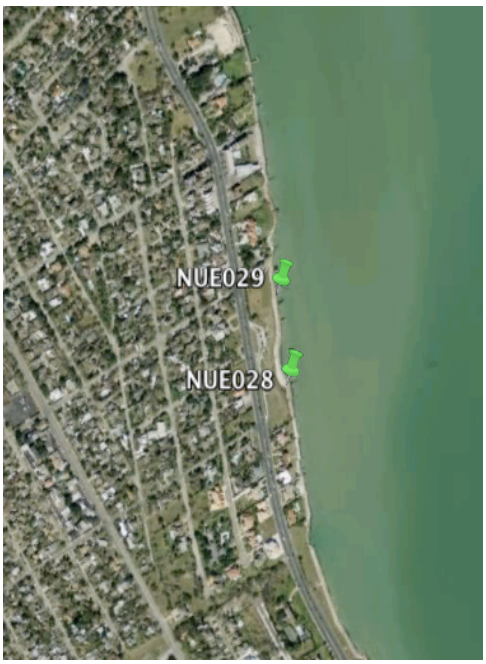

Fig. 2 Satellite image of Ropes Park sampling locations NUE028 and NUE029. Sampling sites are marked.

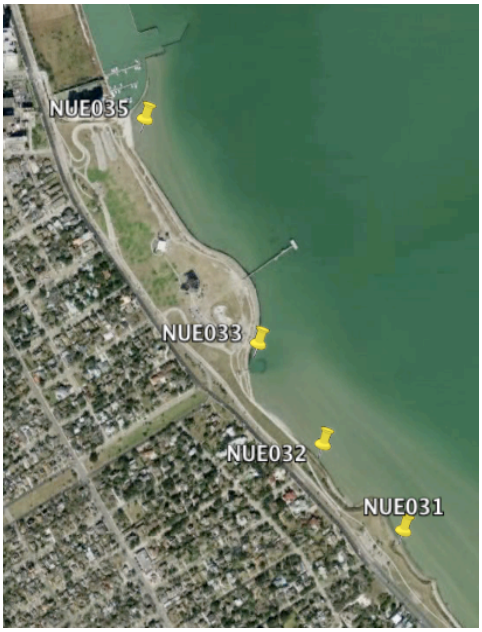

Fig. 3 Satellite image of Cole Park sampling locations NUE031, NUE032, NUE033, and NUE035. Sampling sites are marked.

Table 1 List of sampling sites and GPS coordinates

Sampling site	GPS coordinates (latitude longitude)
NUE028	27°45’11” -97°22’33.4”
NUE029	27°45’17” -97°22’34.4”
NUE031	27°46’04” -97°23’05.2”
NUE032	27°46’12” -97°23’14.0”
NUE033	27°46’18” -97°23’18.0”
NUE035	27°46’35” -97°23’28.1”
OST18499	27°46'59.70" -97°35'32.75"
OST18501	27°42'33.70" -97°33'15.19"
OST18500	27°43'46.09" -97°31'24.85"
OST20198	27°43'50.09" - 97°34'37.12"
OST20559	27°48'0.22" -97°38'47.51"

Sample Collection and *Enterococcus* Enumeration

Water samples from Ropes and Cole parks were collected over an eight month period with sampling events scheduled monthly and additional sampling events following rainfall. A rainfall event was defined as at least 2.5 cm of rain in a 24 hour period or greater than 7.5 cm of rain in a seven day period, according to an online weather site (www.wunderground.com). Oso Creek samples were collected as part of the regular quarterly sampling of water under the TSSWCB project, at the five stations, for enterococci analysis. Rainfall was recorded for these sampling events, but the events were planned regardless of rainfall or dry weather. Three water samples per site were collected for each sampling event from the six TBW stations at Cole and Ropes parks and

five TSSWCB stations in accordance with guidelines specified in the current TBW QAPP and the TSSWCB QAPP (Mott and Hay 2010). However, instead of one liter bottles, two liter bottles were used for Ropes and Cole parks in order to obtain enough water for additional assays performed for a separate project. Salinity, pH, temperature, and dissolved oxygen were measured in the field with a YSI multiprobe instrument. Turbidity was measured in the laboratory with a turbidimeter (model DRT-15CE, HF Scientific). Water samples were transported to the lab at 4°C within six hours of collection. Enterococci were enumerated using membrane filtration and plating following USEPA Method 1600, consistent with procedures from the TBW QAPP and the TSSWCB QAPP (USEPA 2002; Mott and Hay 2010). *Enterococcus* counts were determined after 24 hours incubation.

esp Analysis

Procedures for *esp* analysis followed analytical procedures in a QAPP approved by the TSSWCB and USEPA (Mott and Hay 2010) following a protocol provided by Dr. K. Gordon and Dr. V.J. Harwood, University of South Florida (Appendix A). Environmental water (300 ml) was filtered through 0.45 µm nitrocellulose filters. The filters were placed on membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) and incubated at 41°C for 48 hours. The filters were then transferred to 15 ml tubes containing five ml azide dextrose broth (Difco) and incubated for three hours on a shaking table at 41°C. Following enrichment in the azide dextrose broth, DNA extraction was performed with the Qiagen™ DNeasy Blood and Tissue Kit and ASL buffer (Qiagen, Inc.). Initial results suggested that the polymerase chain reaction (PCR) was being inhibited by factors in the environmental water samples, so for the remaining

samples, InhibitEX™ tablets (Qiagen, Inc.) were used during extraction to prevent inhibition.

The PCR reaction mix was prepared using GoTaq Green mix (Promega) according to the manufacturer's instruction, with *esp* forward primer (5'-TAT GAA AGC AAC AGC ACA AGT T-3') (Scott *et al*. 2005) and *esp* reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3') (Hammerum and Jensen 2002). Each PCR experiment contained a minimum of six controls, described below, and three samples from each of the sample sites. Agarose gel electrophoresis with a Promega 100 bp ladder was used to visualize the PCR product with a positive result indicated by a band at the 680 bp mark. Details for the entire *esp* protocol, including PCR conditions, are included in Appendices A and B.

Controls for *esp* Analysis

Controls for *esp* anlysis included the following, at various steps in the process:

1. a method blank (MB) (300 ml sterile buffered dilution water) carried in the field and processed in the same manner as the water samples to demonstrate a lack of contamination during sample collection
2. a method spike, SP2, (300 ml sterile buffered dilution water spiked with 100 µl of an overnight culture of *Enterococcus faecium* C68, which contains the *esp* gene) filtered through a 0.45 µm nitrocellulose filter
3. an inhibition spike, SP1, (300 ml composite sample [all environmental samples mixed together] spiked with 100 µl of an overnight culture of *E. faecium* C68, which contains the *esp* gene) filtered through a 0.45 µm nitrocellulose filter

SP1, SP2, and MB were carried through the entire analysis, from sample processing to electrophoresis. The purpose of SP1 and SP2 was to check for PCR inhibitors present in the environmental samples. A positive result for both SP1 and SP2 indicated no inhibition. Inhibition was suspected when SP2 was positive and SP1 was negative. Once PCR inhibition was suspected, additional PCR reactions from each site were spiked with *E. faecium* C68 DNA to determine which site analyses were experiencing inhibition.

4. an extraction blank (EB): DNA extraction performed without adding any additional culture or sample water, thus serving as a negative control for the extraction process
5. +PCR: positive control for PCR using *Enterococcus faecium* C68 DNA
6. -PCR: negative control for PCR; no DNA added to reaction

The PCR controls provided assurance that the PCR protocol had been followed correctly and detection of the *esp* gene was possible.

Identification of *Enterococcus* spp.

In order to determine whether *Enterococcus faecium* was present at the sampling sites, a subset of water isolates collected from Ropes and Cole parks following rainfall (10/22/10) were speciated using the MicroLog™ Microbial Identification System (MIS) (Biolog, Inc.) following the MicroLog™ System Release 4.0 User Guide (Biolog 1999). Each isolate was transferred to a Biolog™ Universal Growth (BUG B) plate with five percent sheep's blood agar (Biolog 1999). The plates were incubated at 35°C for 16 h. Growth from the plates was transferred to inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, 0.01% Phytagel™) to reach a transmittance level of 20% (± 2%) at 600nm. This

inoculum was pipetted into a 96 well GP2 Microplate™, and the plates were incubated for 17 h at 35°C. After the incubation period the plates were read using the Micro Log System™, Release 4.20.04 (Biolog 2004). The following bacteria were used as controls for the MicroLog™ MIS: *Corynebacterium minutissimum* ATCC strain 23348, *Rhodococcus equi* ATCC strain 6929, *Staphylococcus aureus* ATCC strain 12600, and *Enterococcus faecalis* ATCC strain 19433.

RESULTS

Environmental Conditions

The temperature of the marine water taken at Ropes and Cole parks, measured in the field with the YSI multiprobe instrument, ranged from 12.0°C in the winter (2/17/10) to 31.4°C in the summer (7/6/10). Salinity ranged from 26.2 ppt to 33.7 ppt, and pH ranged from 6.5 to 8.2.

The temperature of the freshwater from Oso Creek ranged from 12.2°C in December (12/6/10) to 32.2°C in September (9/13/10). The highest salinity recorded was 3.6 ppt on 10/18/10 at TCEQ site OST18500. OST18500 had the highest average salinity of 2.3 ppt. The freshwater samples had a pH ranging from 7.2 to 8.1. The environmental conditions recorded at each site are listed by date and sample site in Appendix D.

esp Analysis of Marine Water Samples Following Dry Weather

The *esp* gene was not detected in any of the marine samples collected from February through July 2010 (Table 2). *Enterococcus* levels were relatively low at most stations for the dry weather events during this time period, with less than 30 CFU 100 ml^{-1} for 23 of the 36 sample averages and exceeding 500 CFU 100 ml^{-1} only on 6/3/10 at NUE028, NUE031, and NUE032 (Figure 4), so levels of *E. faecium* would also be expected to be low. In February, all control results were as expected; *esp* was detected in SP1, SP2, and +PCR, but it was not detected in MB, EB, or -PCR. These findings indicated that the assay was able to detect *esp*, and inhibition was not occurring (Figures 5 and 6).

Table 2 Results from marine water at Ropes and Cole parks following dry weather

Sample number	2/17/10 Ent* CFU 100 ml^{-1}	*esp*	3/24/10 Ent* CFU 100 ml^{-1}	*esp*	4/28/10 Ent* CFU 100 ml^{-1}	*esp*	5/26/10 Ent* CFU 100 ml^{-1}	*esp*	6/3/10 Ent* CFU 100 ml^{-1}	*esp*	7/6/10 Ent* CFU 100 ml^{-1}	*esp*
NUE028A		-		-		-		-		-		-
NUE028B	4.3 ± 0.9	-	323.3 ± 52.1	-	23.3 ± 1.7	-	10.3 ± 3.9	-	566.7 ± 110.5	-	6 ± 1.5	-
NUE028C		-		-		-		-		-		-
NUE029A		-		-		-		-		-		-
NUE029B	5.7 ± 2.2	-	271 ± 45.4	-	41 ± 4.9	-	10 ± 1.7	-	220 ± 26.5	-	5.7 ± 1.5	-
NUE029C		-		-		-		-		-		-
NUE031A		-		-		-		-		-		-
NUE031B	16 ± 3.2	-	81.3 ± 3.0	-	6.3 ± 0.7	-	3 ± 1.5	-	1166.7 ± 101.7	-	13.7 ± 3.5	-
NUE031C		-		-		-		-		-		-
NUE032A		-		-		-		-		-		-
NUE032B	5 ± 0.6	-	110.3 ± 17.6	-	2.7 ± 0.3	-	1.3 ± 0.3	-	900 ± 20.1	-	1 ± 0.6	-
NUE032C		-		-		-		-		-		-
NUE033A		-		-		-		-		-		-
NUE033B	5.3 ± 1.2	-	27.7 ± 3.7	-	1.7 ± 0.3	-	51.7 ± 23.1	-	77.7 ± 3.9	-	12 ± 3.1	-
NUE033C		-		-		-		-		-		-
NUE035A		-		-		-		-		-		-
NUE035B	4.7 ± 1.9	-	154 ± 13.4	-	25.3 ± 5.4	-	3.7 ± 0.7	-	37.3 ± 8.8	-	10.7 ± 0.3	-
NUE035C		-		-		-		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).

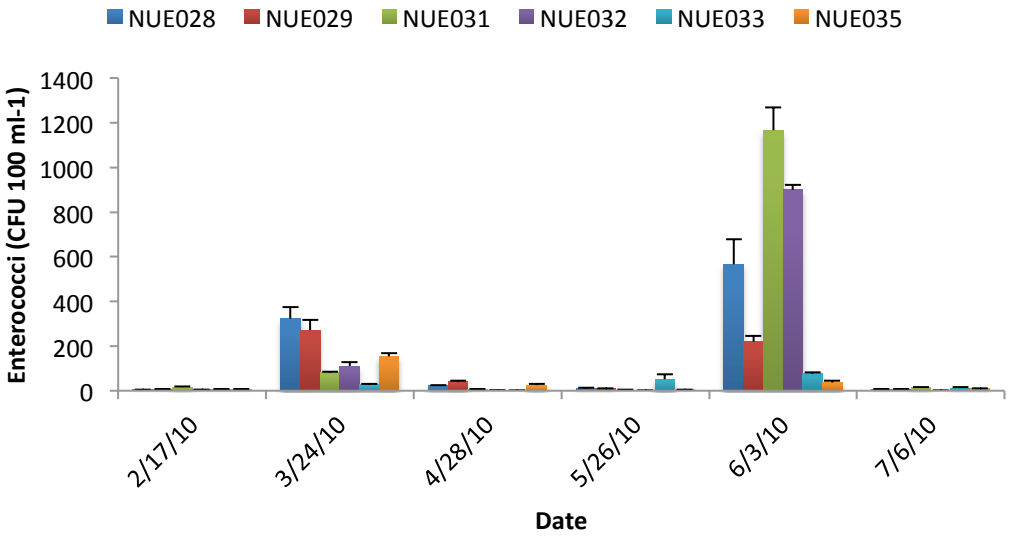

Fig. 4 *Enterococcus* concentrations at Ropes and Cole parks following dry weather. Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

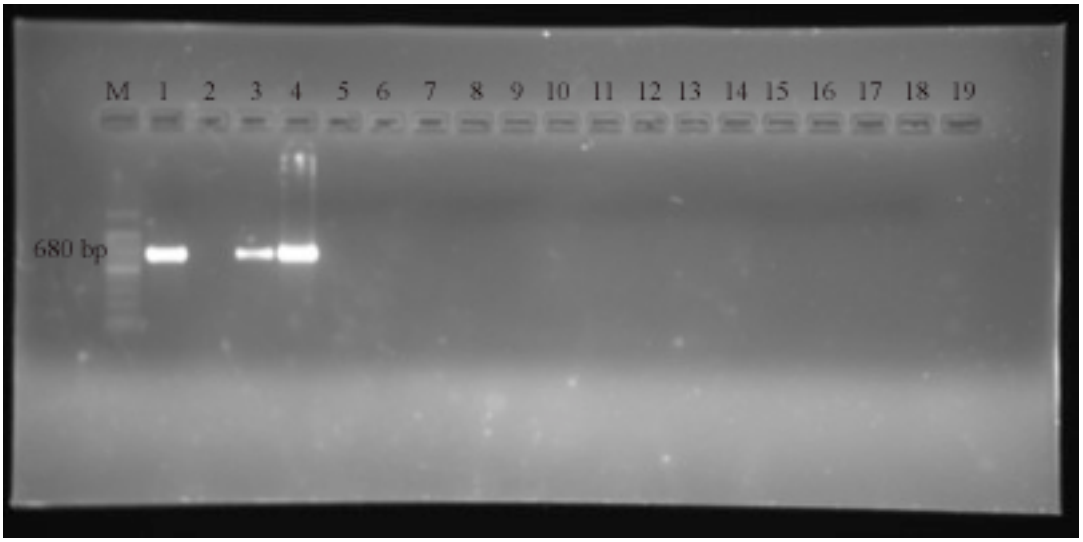

Fig. 5 February agarose gel electrophoresis following dry weather. Samples were collected on 2/17/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples on 2/22/10 and 2/23/10. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 NUE028A, 8 NUE028B, 9 NUE028C, 10 NUE029A, 11 NUE029B, 12 NUE029C, 13 NUE031A, 14 NUE031B, 15 NUE031C, 16 NUE032A, 17 NUE032B, 18 NUE032C, 19 Empty.

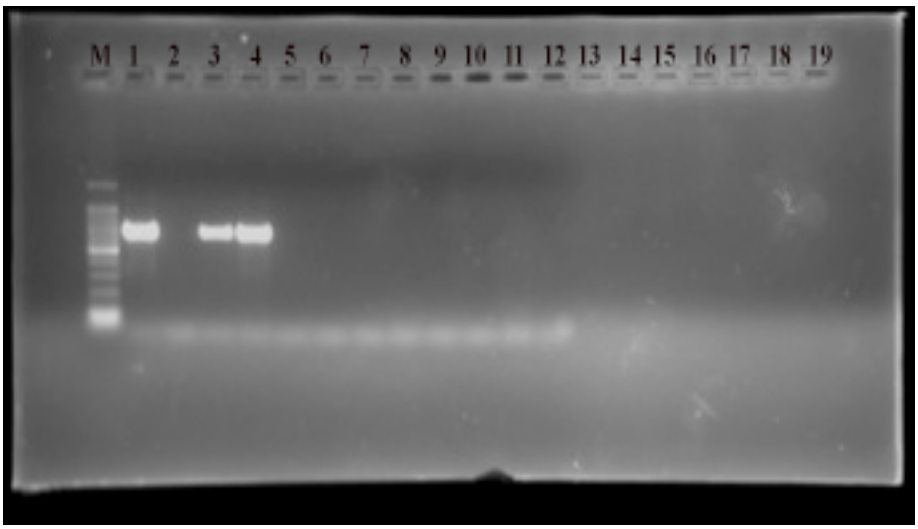

Fig. 6 February agarose gel electrophoresis following dry weather continued. Samples were collected on 2/17/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples on 2/22/10 and 2/23/10. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 NUE033A, 8 NUE033B, 9 NUE033C, 10 NUE035A, 11 NUE035B, 12 NUE035C, 13-19 Empty.

In March, +PCR was positive for *esp.* SP1, SP2, all negative controls, and all environmental samples were negative for *esp.* There may have been a problem with the *Enterococcus faecium* C68 culture used for filtration (Figure 7). Inhibition was suspected in April because the dilution water spikes (SP2) were positive, and the composite spikes (SP1) were negative (Figure 8). Other controls were as expected. In May, +PCR was positive for *esp,* and -PCR was negative for *esp* (Figure 9). However, both the method blank and extraction blank showed very faint bands, indicating possible contamination during analysis. SP1and all environmental samples were negative for *esp.* The negative composite sample indicates that inhibition may have occurred. The positive results for

the negative controls do not affect the reliability of these results because no samples were positive for *esp*. A positive result for negative controls indicates the possibility of false positives, not false negatives.

Fig. 7 March agarose gel electrophoresis following dry weather. Samples were collected on 3/24/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 NUE028A, 8 NUE028B, 9 NUE028C, 10 NUE029A, 11 NUE029B, 12 NUE029C, 13 NUE031A, 14 NUE031B, 15 NUE031C, 16-19 Empty, 20 C68 (positive PCR control), 21 Negative PCR control, 22 SP1 (composite sample with C68), 23 SP2 (dilution water with C68), 24 Method Blank (dilution water), 25 Extraction Blank, 26 Empty, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A, 32 NUE033B, 33 NUE033C, 34 Empty, 35 NUE035A, 36 NUE035B, 37 NUE035C, 38 Empty.

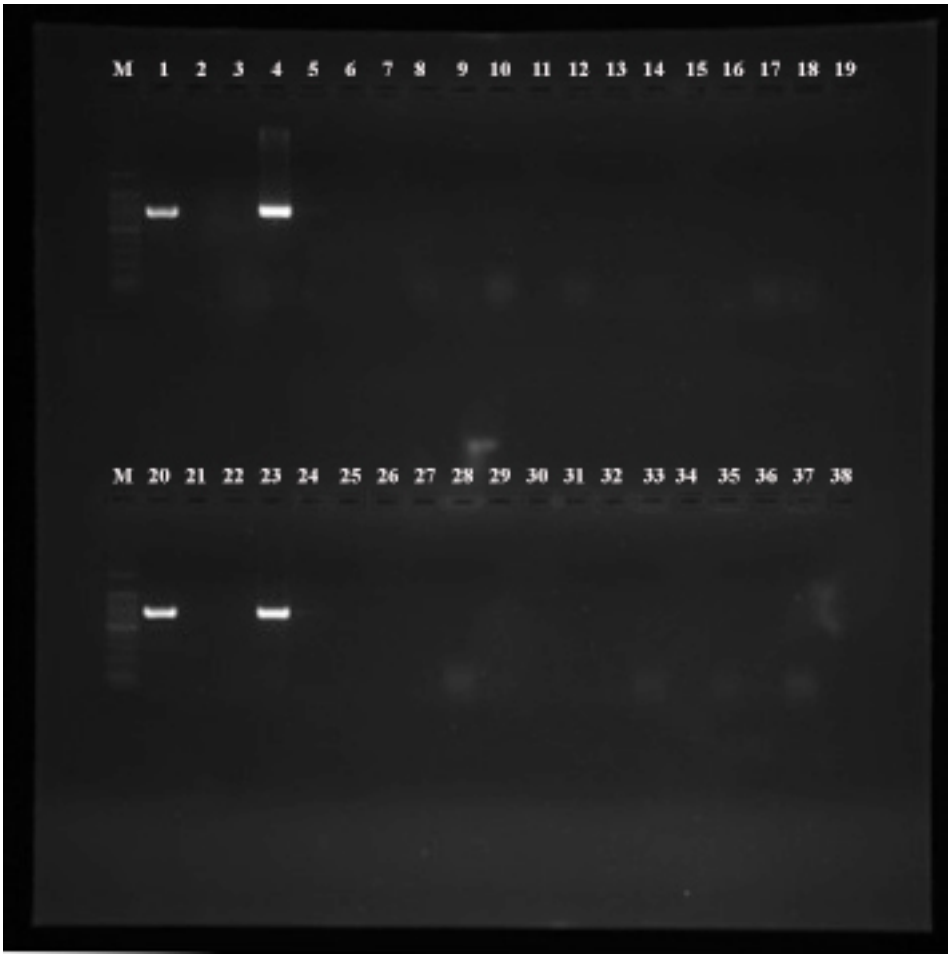

Fig. 8 April agarose gel electrophoresis following dry weather. Samples were collected on 4/28/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A, 9 NUE028B, 10 NUE028C, 11 Empty, 12 NUE029A, 13 NUE029B, 14 NUE029C, 15 Empty, 16 NUE031A, 17 NUE031B, 18 NUE031C, 19 Empty, 20 C68 (positive PCR control), 21 Negative PCR control, 22 SP1 (composite sample with C68), 23 SP2 (dilution water with C68), 24 Method Blank (dilution water), 25 Extraction Blank, 26 Empty, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A, 32 NUE033B, 33 NUE033C, 34 Empty, 35 NUE035A, 36 NUE035B, 37 NUE035C, 38 Empty.

Fig. 9 May agarose gel electrophoresis following dry weather. Samples were collected on 5/26/10 and filtered through a 0.45 μm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A, 9 NUE028B, 10 NUE028C, 11 Empty, 12 NUE029A, 13 NUE029B, 14 NUE029C, 15 Empty, 16 NUE031A, 17 NUE031B, 18 NUE031C, 19 Empty, 20 C68 (positive PCR control), 21 Negative PCR control, 22 SP1 (composite sample with C68), 23 SP2 (dilution water with C68), 24 Method Blank (dilution water), 25 Extraction Blank, 26 Empty, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A, 32 NUE033B, 33 NUE033C, 34 Empty, 35 NUE035A, 36 NUE035B, 37 NUE035C, 38 Empty.

To further identify for which sites the protocol was affected by inhibition, an additional reaction for each site was spiked with C68 DNA as recommended in the literature (Ahmed *et al*. 2008). In June and July, one sample from each site at Ropes and Cole parks was analyzed with and without the addition of *E. faecium* C68 DNA to the PCR chamber; inhibition was evident for every site except NUE033 in June and for every site in July (Figures 10 and 11). In both months, +PCR and SP2 were positive for *esp,* and -PCR, SP1, EB, and MB were negative for *esp*, To avoid inhibition in September, inhibitEX tablets (Qiagen) were used during DNA extraction, according to the protocol for inhibitory sites (Appendix B) and results of controls showed that this treatment was effective in the elimination of inhibition. Each sample spiked with C68 DNA and extracted using the protocol for inhibitory sites showed up as a bright band on the agarose gel (Figure 12).

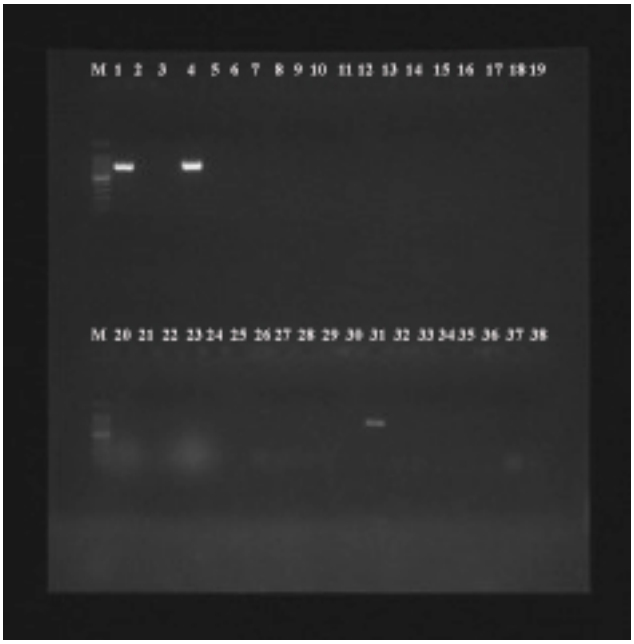

Fig. 10 June agarose gel electrophoresis following dry weather. Samples were collected on 6/03/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A with C68, 9 NUE028A, 10 NUE028B, 11 NUE028C, 12 Empty, 13 NUE029A with C68, 14 NUE029A, 15 NUE029B, 16 NUE029C, 17-19 Empty, 20 Empty, 21 NUE031A with C68, 22 NUE031A, 23 NUE031B, 24 NUE031C, 25 Empty, 26 NUE32A with C68, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A with C68, 32 NUE033A, 33 NUE033B, 34 NUE033C, 35 NUE035A with C68, 36 NUE035A, 37 NUE035B, 38 NUE035C.

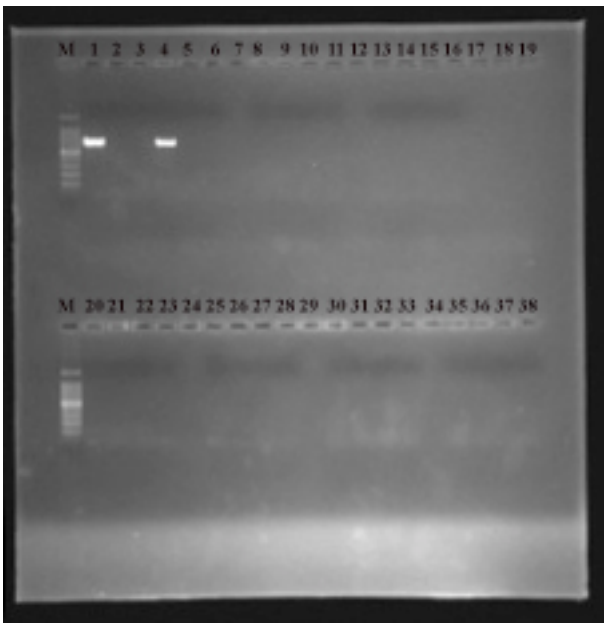

Fig. 11 July agarose gel electrophoresis following dry weather. Samples were collected on 7/06/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A with C68, 9 NUE028A, 10 NUE028B, 11 NUE028C, 12 Empty, 13 NUE029A with C68, 14 NUE029A, 15 NUE029B, 16 NUE029C, 17-19 Empty, 20 Empty, 21 NUE031A with C68, 22 NUE031A, 23 NUE031B, 24 NUE031C, 25 Empty, 26 NUE32A with C68, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A with C68, 32 NUE033A, 33 NUE033B, 34 NUE033C, 35 NUE035A with C68, 36 NUE035A, 37 NUE035B, 38 NUE035C.

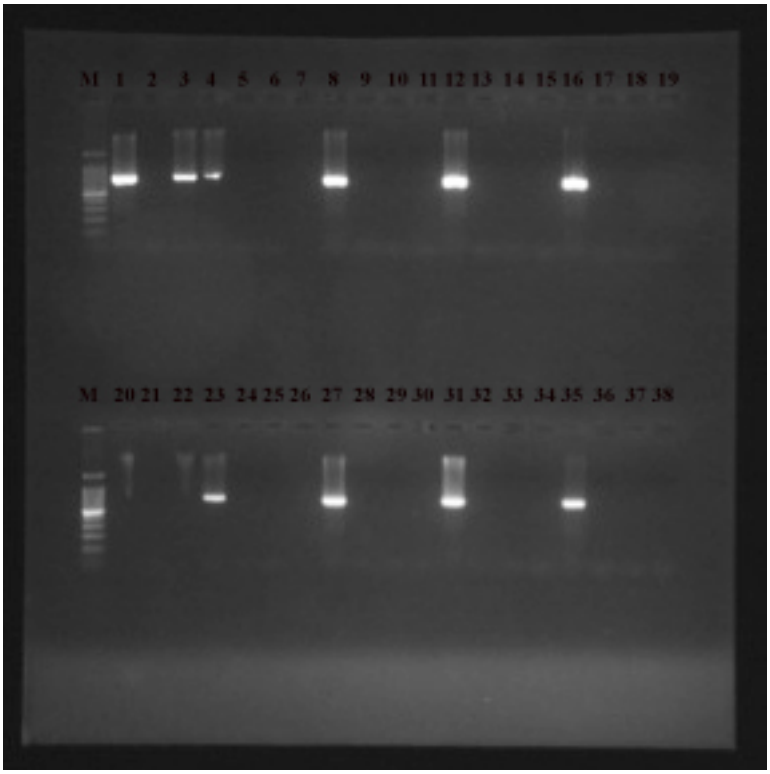

Fig. 12 September agarose gel electrophoresis following dry weather. Samples were collected on 9/10/10 and filtered through a 0.45 μm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A with C68, 9 NUE028A, 10 NUE028B, 11 NUE028C, 12 NUE029A with C68, 13 NUE029A, 14 NUE029B, 15 NUE029C, 16 NUE031A with C68, 17 NUE031A, 18 NUE031B, 19 NUE031C, 20 C68 (positive PCR control), 21 Negative PCR control,22 SP1 (composite sample with C68), 23 SP2 (dilution water with C68), 24 Method Blank (dilution water), 25 Extraction Blank, 26 Empty, 27 NUE32A with C68, 28 NUE032A, 29 NUE032B, 30 NUE032C, 31 NUE033A with C68, 32 NUE033A, 33 NUE033B, 34 NUE033C, 35 NUE035A with C68, 36 NUE035A, 37 NUE035B, 38 NUE035C.

PCR Inhibition

PCR inhibition was a factor in the first half of this study; the controls from the first sampling event were as expected, but the spike controls in March, April, and May indicated inhibition. A 1:10 dilution of the DNA in the PCR reaction did not solve the inhibition problem, either because the inhibition was still taking place or because the *esp* marker was below the minimum limit of detection. To investigate which sites' analyses might have been affected by inhibition, additional controls were added to the method, as described in a previous study (Ahmed *et al.* 2008). A spiked control for each sample site, in addition to the composite spike, would pinpoint which sites were experiencing inhibition. Unfortunately, all sites were affected by inhibition, so the protocol for inhibitory samples (Appendix B) was used for all analyses after July. This protocol was effective in eliminating PCR inhibition, as evidenced by the individual spike controls.

***esp* Analysis of Marine Water Samples Following Rainfall**

For the rainfall events at Ropes and Cole parks, enterococci levels were less than 500 CFU 100 ml^{-1} except for NUE033 on 5/16/10 and the 9/22/10 event where levels exceeded 1000 CFU/100ml at both Ropes Park stations: NUE028 and NUE029 (Table 3 and Figure 13). The *esp* gene was detected in one sample from NUE028 and two from NUE029, indicating human source contamination at Ropes Park on 9/22/10 (Table 3).

Table 3 Results from marine water at Ropes and Cole parks following rainfall

	5/16/10 (2.5 cm 24 h rainfall)		6/09/10 (3.6 cm 24 h rainfall)		9/10/10 (9.1 cm 7 d rainfall)		9/22/10 (21.0 cm 7 d rainfall)	
Sample number	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*
NUE028 A		-		-		-		+
NUE028 B	60 ± 6.4	-	115.7 ± 1.3	-	46 ± 11.5	-	1603.3 ± 43.7	-
NUE028 C		-		-		-		-
NUE029 A		-		-		-		+
NUE029 B	133.3 ± 21.9	-	286.3 ± 40.8	-	38 ± 2	-	2313.3 ± 700.8	+
NUE029 C		-		-		-		-
NUE031 A		-		-		-		-
NUE031 B	5 ± 1.5	-	232.3 ± 43.8	-	65.3 ± 2.7	-	863.3 ± 94.0	-
NUE031 C		-		-		-		-
NUE032 A		-		-		-		-
NUE032 B	5.7 ± 0.9	-	116.7 ± 21.9	-	37.7 ± 3.2	-	620 ± 20	-
NUE032 C		-		-		-		-
NUE033 A		-		-		-		-
NUE033 B	2315.7 ± 368.5	-	131.3 ± 18.4	-	217 ± 33.1	-	710 ± 34.6	-
NUE033 C		-		-		-		-
NUE035 A		-		-		-		-
NUE035 B	30 ± 1.2	-	67 ± 39.5	-	389 ± 126.0	-	756.7 ± 80.9	-
NUE035 C		-		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).

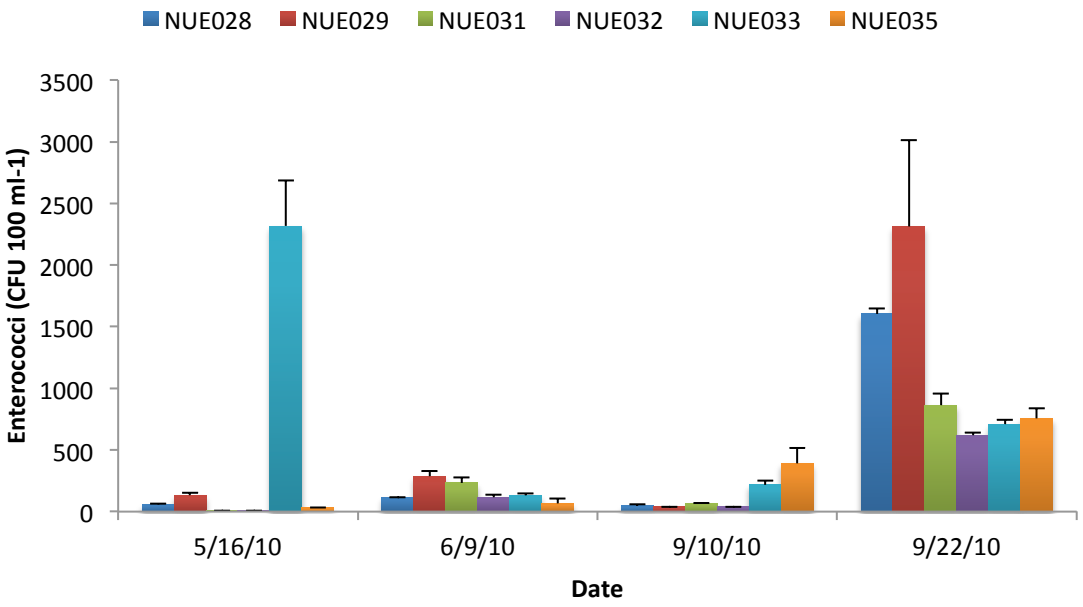

Fig. 13 *Enterococcus* concentrations at Ropes and Cole parks following rainfall. Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

In May, +PCR and SP1 were positive for *esp*. All negative controls (-PCR, MB, and EB) were negative for *esp*. SP2 was negative for *esp,* but this was not unexpected because growth was very limited on the filter. Environmental samples were negative for *esp* (Figures 14 and 15). In June, the composite spike (SP1) was negative while the spike in dilution water (SP2) was positive for *esp* (Figure 16). To test for inhibition at each site individually, C68 genomic DNA was added to a separate PCR chamber for each of the A samples. Each of these spikes was negative for *esp*, indicating that inhibition had occurred at all sites, similar to the results from dry weather samples. All negative controls (-PCR, MB, and EB) were negative for *esp*. All environmental samples were negative for *esp*. In September, the protocol for inhibitory samples (Appendix B) was effective in

eliminating inhibition of PCR (Figure 17). SP1 and SP2 were positive for *esp*. Each of the C68 spikes was positive for *esp.* All negative controls were negative for *esp*: -PCR, MB, and EB. NUE028A was positive for *esp*. NUE029A and NUE029B were positive for *esp.* All other sites were negative for *esp.*

Fig. 14 May agarose gel electrophoresis following rainfall. Samples were collected on 5/16/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A, 9 NUE028B, 10 NUE028C, 11 Empty, 12 NUE029A, 13 NUE029B, 14 NUE029C, 15 Empty, 16 NUE031A, 17 NUE031B, 18 NUE031C, 19 Empty.

Fig. 15 May agarose gel electrophoresis following rainfall continued. Samples were collected on 5/16/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE032A, 9 NUE032B, 10 NUE032C, 11 Empty, 12 NUE033A, 13 NUE033B, 14 NUE033C, 15 Empty, 16 NUE035A, 17 NUE035B, 18 NUE035C, 19 Empty.

Fig. 16 June agarose gel electrophoresis following rainfall. Samples were collected on 6/09/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for non-inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A with C68, 9 NUE028A, 10 NUE028B, 11 NUE028C, 12 Empty, 13 NUE029A with C68, 14 NUE029A, 15 NUE029B, 16 NUE029C, 17-19 Empty, 20 Empty, 21 NUE031A with C68, 22 NUE031A, 23 NUE031B, 24 NUE031C, 25 Empty, 26 NUE32A with C68, 27 NUE032A, 28 NUE032B, 29 NUE032C, 30 Empty, 31 NUE033A with C68, 32 NUE033A, 33 NUE033B, 34 NUE033C, 35 NUE035A with C68, 36 NUE035A, 37 NUE035B, 38 NUE035C.

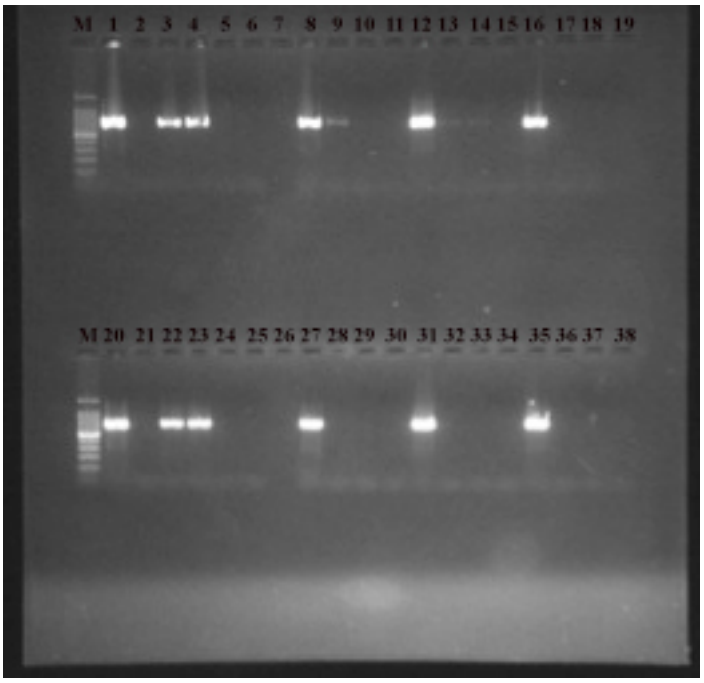

Fig. 17 September agarose gel electrophoresis following rainfall. Samples were collected on 9/22/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 Empty, 8 NUE028A with C68, 9 NUE028A, 10 NUE028B, 11 NUE028C, 12 NUE029A with C68, 13 NUE029A, 14 NUE029B, 15 NUE029C, 16 NUE031A with C68, 17 NUE031A, 18 NUE031B, 19 NUE031C, 20 C68 (positive PCR control), 21 Negative PCR control,22 SP1 (composite sample with C68), 23 SP2 (dilution water with C68), 24 Method Blank (dilution water), 25 Extraction Blank, 26 Empty, 27 NUE32A with C68, 28 NUE032A, 29 NUE032B, 30 NUE032C, 31 NUE033A with C68, 32 NUE033A, 33 NUE033B, 34 NUE033C, 35 NUE035A with C68, 36 NUE035A, 37 NUE035B, 38 NUE035C.

Identification of *Enterococcus* spp.

After the rainfall event on 9/22/10, 140 colonies were isolated from filters on mEI from all five sampling sites at Ropes and Cole parks. Ten colonies from each site were spread on mEI, and the rest of the colonies were grown for six or more hours in tryptic soy broth (TSB). Of the 140 colonies, 58 were able to grow on mEI or in TSB and were each inoculated into 96 well GP2 Microplates™ (Biolog, Inc.). Of the 58 isolates, 47 were identified to at least genus level, and 36 isolates were identified to the species level (Table 4 and Appendix C).

Table 4 Identification of *Enterococcus* spp. following rainfall at Ropes and Cole parks

Sampling site	# Identified isolates*	# *Enterococcus*	# *E. faecium*	Other genera identified*	Other species identified*
NUE028	11	9	2	*Pediococcus* *Streptococcus*	*E. mundtii* *E. casseliflavus* *S. criceti*
NUE029	7	6	4	*Pediococcus*	*E. mundtii* *P. acidilactici/parvulus* *E. hirae*
NUE031	10	7	4	*Pediococcus* *Alloiococcus* *Streptococcus*	*E. dispar* *A. otitis* *E. faecalis* *S. hyointestinalis* *E. gallinarum*
NUE032	5	5	0	none	*E. casseliflavus* *E. solitarius*
NUE033	10	6	2	*Pediococcus* *Alloiococcus*	*E. faecalis* *P. pentosaceus* *P. acidilactici/parvulus* *A. otitis* *E. casseliflavus*
NUE035	4	3	1	*Pediococcus*	*P. pentosaceus* *E. casseliflavus* *E. gallinarum*
TOTAL	47	36	13	3	12

*A similarity index (SIM) between 0.5 and 1.0 is required for an isolate to be considered identified, for both genus identification and species identification.

***esp* Analysis of Freshwater Samples**

The *esp* marker was detected in freshwater at three of the five Oso Creek TCEQ sites over the course of this study (Tables 5 – 7). InhibitEX™ tablets (Qiagen) were used according to the protocol for inhibitory samples (Appendix B) for each sampling event. Beginning with the samples from 9/13/10, additional PCR controls were added to test individual sites for inhibition; one sample from each site was analyzed with and without the addition of *E. faecium* C68 DNA to the PCR chamber. Each of these spikes tested positive for *esp*, indicating that inhibition was not occurring, except for the sample from OST18501 on 9/13/10 (Figure 19).

Table 5 Results from freshwater collected from Oso Creek at historic TCEQ sites in 2010

	7/07/10		9/13/10		10/18/10		12/06/10	
Sample number	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*
18499A		-		-		-		-
18499B	988.3 ± 61.9	-	405.7 ± 81.9	-	513.3 ± 121.4	+	978 ± 160.2	-
18499C		-		-		-		-
18500A		-		-		-		-
18500B	1477.3 ± 219.1	-	1088.7 ± 178.9	-	983.3 ± 37.2	-	363.3 ± 31.8	-
18500C		-		-		-		-
18501A		-		-		-		-
18501B	1044 ± 174.4	-	426.7 ± 43.3	-	423.3 ± 95.3	-	383.3 ± 235.5	-
18501C		-		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).

Table 6 Results from freshwater collected from Oso Creek at historic TCEQ sites in 2011

	1/19/11		3/09/11		4/20/11	
Sample number	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100ml^{-1}	*esp*
18499A		-		-		-
18499B	3188.7 ± 433.0	-	633.3 ± 117.2	-	152.3 ± 14.7	-
18499C		-		-		-
18500A		-		-		+
18500B	2600 ± 96.4	-	503.3 ± 48.4	-	572 ± 36.0	+
18500C		-		-		+
18501A		-		-		-
18501B	3189 ± 48.5	-	97 ± 7.4	-	19.3 ± 8.7	-
18501C		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).

Table 7 Results from freshwater collected from Oso Creek at TCEQ sites OST20198 and OST20559

	9/13/10		12/06/10		3/09/11	
Sample number	Ent* CFU 100ml^{-1}	*esp*	Ent* CFU 100ml^{-1}	*esp*	Ent* CFU 100ml^{-1}	*esp*
20198A		-				
20198B	8233.3 ± 2281.3	-	DRY†		DRY†	
20198C		-				
20559A		-		-		-
20559B	317.7 ± 29.9	-	89.7 ± 6.7	+	191 ± 18.2	-
20559C		-		-		+

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).
† The sample location was dry, so water could not be collected.

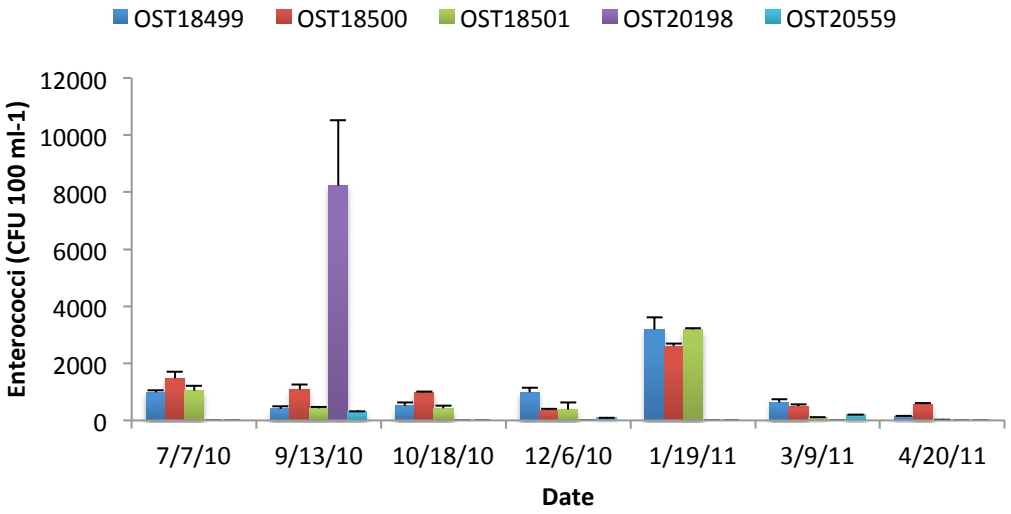

Fig. 18 *Enterococcus* concentrations in Oso Creek at TCEQ sites. Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

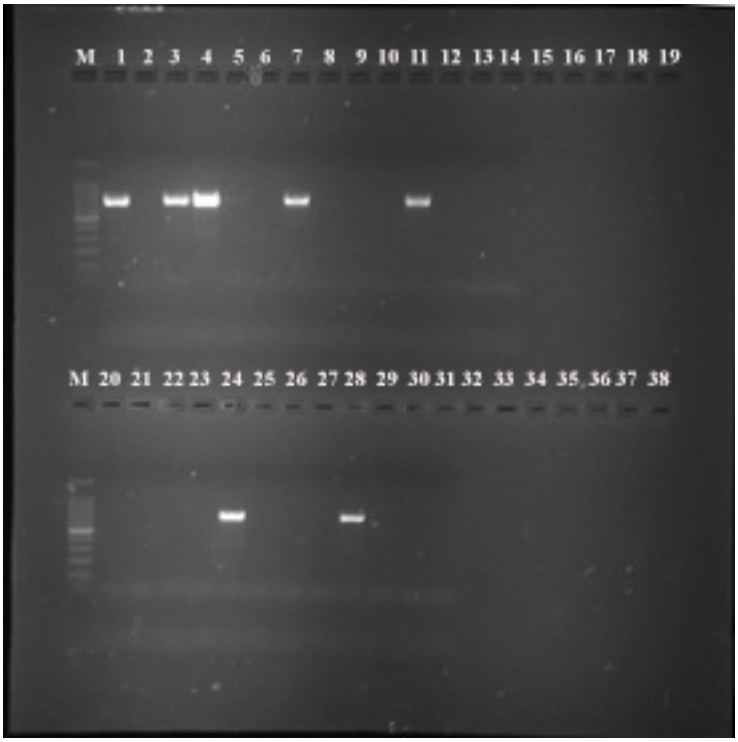

Fig. 19 September agarose gel electrophoresis of freshwater samples. Samples were collected on 9/13/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 18499A with C68, 8 18499A, 9 18499B, 10 18499C, 11 18500A with C68, 12 18500A, 13 18500B, 14 18500C, 15-19 Empty, 20 18501A with C68, 21 18501A, 22 18501B, 23 18501C, 24 20559A with C68, 25 20559A, 26 20559B, 27 20559C, 28 20198A with C68, 29 20198A, 30 20198B, 31 20198C, 32-38 Empty.

All environmental samples were negative for *esp* on 7/7/10 (Table 5). The *Enterococcus* concentrations ranged from 700 to 1900 CFU 100 ml^{-1} (Figure 18). All environmental samples from 9/13/10 were negative for the marker as well, with *Enterococcus* concentrations ranging from 300 to 12,500 CFU 100 ml^{-1}(Tables 5 and 7). Although these concentrations are high, the absence of *esp* was not unexpected. The creek runs through farm and ranch pastures in addition to being subjected to urban runoff; thus, the high bacteria concentrations could easily be attributed to animals. On 10/18/10, one sample from OST18499 tested positive for *esp* (Figure 20). The *Enterococcus* concentration for this sample was 310 CFU 100 ml^{-1} (Table 5). One sample from OST20559, located downstream of the Robstown wastewater treatment facility, was positive for *esp* on 12/6/10. The *Enterococcus* concentration for this sample was 83 CFU 100 ml^{-1} (Table 7). Another sample from this site tested positive for the marker on 3/9/11 with an *Enterococcus* concentration of 160 CFU 100 ml^{-1} (Table 7). The *esp* gene was detected in all three samples from OST1500 on 4/20/11. The *Enterococcus* concentration at this site ranged from 500 to 608 with a mean of 572 ± 36 CFU 100 ml^{-1} (Figure 18).

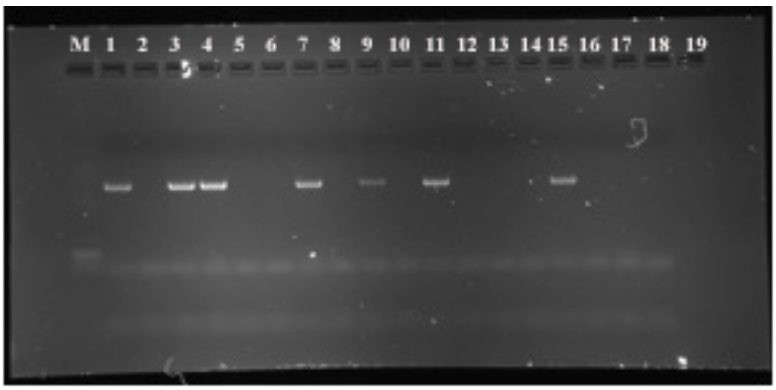

Fig. 20 October agarose gel electrophoresis of freshwater samples. Samples were collected on 10/18/10 and filtered through a 0.45 µm nitrocellulose filter. The filter was placed on (mEI) and incubated at 41°C for 48 hours. DNA extractions and PCR of cultures were performed according to *Enterococcus esp* assay protocol for inhibitory samples. Lanes: M DNA ladder, 1 C68 (positive PCR control), 2 Negative PCR control, 3 SP1 (composite sample with C68), 4 SP2 (dilution water with C68), 5 Method Blank (dilution water), 6 Extraction Blank, 7 18499A with C68, 8 18499A, 9 18499B, 10 18499C, 11 18500A with C68, 12 18500A, 13 18500B, 14 18500C, 15 18501A with C68, 16 18501A, 17 18501B, 18 18501C, 19 Empty.

DISCUSSION

The detection of *esp* in Corpus Christi Bay and the Oso Creek watershed indicates that human fecal contamination has contributed to bacterial loading in marine and fresh coastal waters in the Coastal Bend of south Texas. The *esp* marker was detected at two of the six sampling sites at Ropes and Cole parks and at three of the five sampling sites in the Oso Creek watershed. The lowest enterococci concentration of a positive sample was 83 CFU 100 ml^{-1}, from a freshwater sample, which is much lower than the enterococci concentrations of positive samples in similar studies (Scott *et al.* 2005; McDonald *et al*. 2006; Ahmed *et al*. 2008; Korajkic *et al.* 2009). The *esp* marker was only detected in the marine waters on 9/22/10 after an extended period of very heavy rainfall, 21.0 cm in the seven days preceding collection. With *Enterococcus* levels of 603.3 ± 43.7 CFU 100 ml^{-1} and 2313.3 ± 700.8 CFU 100 ml^{-1}g, sites NUE028 and NUE029 greatly exceeded the single sample limit of 104 CFU 100 ml^{-1} in marine or ocean water allowed by the USEPA. However, the marker was not always detected in samples with high fecal indicator bacteria levels. A concurrent study of the Ropes and Cole parks marine water samples targeting molecular markers for human polyomavirus, *Methanobrevibacter smithii*, and *Bacteroides* sp showed no statistically significant relationship between *Enterococcus* concentrations and the detection of these three human sewage markers (Gordon *et al.* 2013). This contrasted with the other four regions of the United States Gulf Coast in Gordon's study, which had a significant association between elevated enterococci levels and detection of sewage markers in surface waters (Gordon *et al.* 2013). The absence of human associated markers in samples with high levels of *Enterococcus* indicates that the source of the bacteria was not human; there are many

alternative sources of these fecal indicator organisms (McQuaig *et al*. 2006; Gordon *et al.* 2013). The high concentrations of *Enterococcus* in the freshwater samples that were negative for *esp* can be explained by the fact that Oso Creek runs through agricultural and ranch land where cattle are present, and thus the enterococci are likely to be of non-human origin.

Although the *esp* marker was detected in only one of the 10 marine sampling events, the presence of the *esp* gene cannot be ruled out during those events because controls showed that PCR inhibition was affecting the *esp* analyses (Figure 8). It is also possible that the *esp* marker was present but in concentrations below the minimum detection limit of this assay. Previous studies have indicated that the limit of detection for this assay is well above the enterococci concentrations of a majority of the marine samples in this study (Scott *et al.* 2005; Ahmed *et al.* 2008). Ahmed *et al.* (2008) determined that 48 ± 7 sewage origin enterococci colonies in a water sample were necessary for *esp* detection, and Scott *et al.* (2005) found that a concentration of 58 ± 24 enterococci CFU 100 ml^{-1} was necessary for *esp* detection. While the *esp* marker was not detected at each site, human contamination was detected using the additional host-specific human molecular markers at least once at each marine sampling site over the course of the study, when *Enterococcus* concentrations were as low as 6 CFU 100 ml^{-1} (Lindsey *et al.* 2011). Microbial source tracking methods targeting markers of the human-associated microbes: human polyomavirus, *Methanobrevibacter smithii*, and *Bacteroides* sp. were able to detect human contamination in 28 samples that were negative for *esp*. While *esp* analysis relies on viable cells which must be cultured, these markers are culture-independent and only test for the presence of specific DNA. Culture independent methods are

advantageous in that they allow for the detection of any known genetic marker rather than only culturable species and may require only a few hours to detect and identify human fecal pollution. However, the presence of DNA does not necessarily mean that viable pathogens are still present and pose a risk to the public. Using both culture dependent and culture independent methods provides a broader understanding of the nature of the pollution.

The *esp* marker was detectable in south Texas coastal waters, but because no single MST method developed to date can identify all pollution sources with 100% accuracy, it is not recommended to use *esp* analysis as the only test for human fecal contamination. Instead, a toolbox approach using a set of MST methods is preferred in order to accurately assess health risk to the public (Ahmed *et al.* 2009; Gordon *et al.* 2013).

The *esp* marker is not present in the feces of all individuals and may vary in concentration (Casarez *et al.* 2007; Field and Samadpour 2007), and the assay has a minimum limit of detection; thus, the lack of a positive result does not completely rule out the presence of human fecal contamination. Employing carbon source utilization arrays, 36 of the 58 isolates identified using MicroLog™ Microbial Identification System (MIS) were from the genus *Enterococcus*; 13 of these were identified as *Enterococcus faecium*. Other genera present included *Pediococcus, Alloiococcus,* and *Streptococcus.* These isolates were collected from filters on mEI from the heavy rainfall event in September. *E. faecium* was present at five of six marine sampling sites, yet *esp* was detected at only two of those sites, NUE028 and NUE029. Further studies in the frequency of the *esp* gene in the human-specific *E. faecium* population of this region, as

well as quality control studies using raw sewage from local sewage treatment plants to determine the limit of detection, would be useful in determining the reliability of this method.

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APPENDIX A

Standard Operating Procedure (Courtesy Dr. K. Gordon)

Enterococcus esp **Assay Protocol for non-inhibitory samples**

Scope of Application

This protocol applies to the extraction of *Enterococcus* spp. DNA from colonies growing on membrane filters incubated on mEI plates from sites where there is no PCR inhibition. DNA is then use to determine the presence of the esp gene at the site(s) in question

Summary

This protocol outlines the correct procedure to handle membranes containing enterococci colonies, as well as how to perform DNA extraction and PCR amplification of the esp gene.

Apparatus/Supplies

- Qiagen DNeasy Blood and Tissue Kit and ASL buffer (purchased separately, Cat #s 69506 and 19082 resp) or
- Qiamp DNA Stool mini kit (not as cost effective as above option)
- Ice cold 100% Ethanol (VWR Cat# EMD-4450)
- 15ml or 50ml centrifuge tube (VWR Cat# 89039-666 and 89039-658)
- 2X GoTaq® Green Master Mix (VWR Cat# PAM7122 Or PAM7123)
- 10 mM esp forward and esp reverse primer
- Nuclease free/ PCR grade water- comes with GoTaq mix or can be purchased separately (Fisher Sci cat # bp2484-50)
- 0.2 ml Thin walled PCR tubes (USA Sci Cat# 1402-8100)
- 0.5 ml Tube (USA Sci Cat# 1605-0099)
- DNA away (VWR cat# 53509-506)
- Thermocycler
- mEI plates
- 0.45 µm nitrocellulose filter
- Azide Dextrose broth (VWR cat# 90003-102)

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation through latex gloves and with inhalation of fumes.

Procedures

When handling filters, it is easy to cross-contaminate samples with the forceps. When possible, a new pair of forceps should be used for each sample. Each pair should be decontaminated by dipping them into reagent alcohol and flaming then dipping into or spraying ends with DNAaway. The DNAaway must then be wiped off with a paper towel. The forceps can then be flamed again prior to use or for added precautions the forceps can be cross-linked in the cross linker, twice at an energy setting of 600.0 (~2 minutes each) before being used to transfer filters. It is especially important that this procedure be followed while transferring the filters from the mEI plates to the azide dextrose tubes.

The spike and method blank (MB) help keep track of cross-contamination; therefore, the MB should always be processed after the spike. The spike should be processed either as the very first 'sample' or after all the samples and before the MB. Samples should be treated in the same order for the whole procedure.

I. Sample Processing (McQuaig *et al.* 2006)

- Filter 300ml of each sample through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

II. Controls

- Prepare two positive controls, each of which will be spiked with 100µl of a 10^{-5} dilution of an overnight culture of *Enterococcus faecium* C68, which contains the *esp* gene. These controls are termed spike 1 (SP1) and spike 2 (SP2). SP1 is a 300 ml composite sample of each of the sites sampled while SP2 is 300 ml of buffered dilution water.
- Prepare a method blank (MB) consisting of 300ml buffered dilution water filtered through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

III. Enrichment Step (McQuaig *et al.* 2006)

Have ready 15 mL screw-cap tubes (1 per sample), each containing 5 ml azide dextrose broth (Difco) [Azide Dextrose broth= 34.7g/L water]. Lift filters containing enterococci colonies from mEI plates with sterile tweezers which have been treated with DNAaway, crumble/fold with the aid of another pair of forceps or a sterile swab, and

place into the top of the tube. Push the filter down with a sterile swab. Vortex vigorously and incubate for 3 hours at 41^{o}C with vigorous shaking to wash bacteria from the filters and enrich the culture.

IMPORTANT NOTE: CHANGE GLOVES FOLLOWING ANY VISIBLE CONTACT WITH LIQUID!! USE BARRIER PIPET TIPS THROUGHOUT!

IV. Preparation for Extraction (with Qiagen DNeasy Blood and Tissue Kit and ASL or the Qiamp DNA Stool mini kit)

- Spray bench with (1) 70% ethanol and wipe, and (2) DNA Away and wipe
- Pre-heat the heating block to 95°C and ensure the 70°C is on and at 70°C.
- Set out and label 1 filter spin column per sample and 1 for extraction control.
- Label 2 sets of 2 ml microcentrifuge tubes for (a) initial centrifugation, and (b) final DNA elution step. These tubes should be crosslinked twice while open in a microcentrifuge tube tray. The tubes should then be closed while still in the crosslinker. You will need one extra tube in each set for the extraction blank.
- Aliquot reagents into 15 ml or 50 ml sterile, screwcap tubes (ASL, AL, ethanol, proteinase K, AW1, AW2, AE) or microcentrifuge tube (proteinase K).

V. Extraction for non-inhibitory sites (Modified from Manufacturers instructions)

- From each sample/control, pipet 1.8 ml into a 2 ml microcentrifuge tube. Excess culture can be stored at 4ºC in case of problems with extraction.
- Centrifuge culture tubes at high speed in microcentrifuge[c] 2-3 min. to pellet.
- Decant the supernatant and resuspend each pellet in 200 µl ASL lysis buffer (Qiagen, Inc.). Also set up the extraction blank (ASL buffer only). Transfer tubes to heating block and incubate at 95ºC for 5 min. Supernatant should be treated as biohazardous waste.
- Add 15 µl of Proteinase K (Qiagen, Inc.) to each sample followed by 200 µl Buffer AL (Qiagen, Inc.).
- Vortex the sample immediately and transfer to 70°C incubator for 10 minutes.
- Add 200 µl ice cold absolute ethanol and vortex the samples immediately.
- Transfer the resulting suspension to filter spin columns, followed by centrifugation at 10,000 × g for 1 min. Remove tubes from centrifuge SLOWLY to avoid wetting the column (this caveat applies to next steps). If the column does get wet just re-centrifuge the sample(s). Discard the collection tube.
- Place columns into new collection tubes and wash each column with 500 µl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. Discard collection tube.

- Place columns into new collection tubes and wash each column with 500 µl buffer AW2 (Qiagen, Inc.) by centrifugation at 14,000 × g for 3 min. VERY CAREFULLY remove from centrifuge and ensure none of the filtrate has splashed back on the column. It is crucial that the column is dry before starting the next step. Discard collection tube.
- Place each column in its CORRESPONDINGLY LABELED MICROCENTRIFUGE TUBE. Add 200 µl buffer AE (Qiagen, Inc.) to the column. Let buffer sit in column 5 min. before centrifugation to maximize yield. Elute DNA by centrifugation at 10,000 × g for 1 min. Store the eluate at -20ºC until used as PCR template.

VI. PCR

- Add template to tubes on bench (NOT under hood) that has been cleaned with DNA Away. Always run a no-DNA PCR negative control in addition to a positive control reaction with *Enterococcus faecium* C68 DNA as the template.

Recipe per reaction using GoTaq Green Mix (Promega; Taq, dNTPs, buffer and gel loading dye included)

- 25 µl GoTaq Green Mix
- 15 µl H_2O
- 2.5 µl *esp* forward primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-TAT GAA AGC AAC AGC ACA AGT T-3'(Scott *et al.* 2005)
- 2.5 µl *esp* reverse primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-ACG TCG AAA GTT CGA TTT CC-3'-(Hammerum and Jensen 2002)
- 5 µl template

PCR Cycle:

- Initial 94°C for 2 min.

30 cycles of:

- 94°C 1 min
- 58°C 1 min
- 72°C 1 min

1 cycle of

- Final 72°C for 5 min

Hold at 4°C

VII. Electrophoresis

- Have ready a 2.0% agarose gel
- Ethidium bromide to stain the DNA can either be added directly to the gel (1ul of 1% EtBR per 50ml of gel) or to the running buffer (20ul of 1% EtBR added to 1L 1X TAE). 1% EtBr solution is a 1%w/v solution in water (eg. 0.1g in 10ml water).
- Load the Promega 100 bp ladder in the first lane (6ul per lane).
- Load 10ul of sample into each lane.
- Run the gel at 90 V for ~ 45 min.
- The expected product is 680 bp.

References

Hammerum, A.M. and Jensen, L.B. (2002) Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark. *J Clin Microbiol* **40**, 4396.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R. and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Appl Environ Microbiol* **72**, 7567-7574.

Scott, T.M., Jenkins, T.M., Lukasik, J. and Rose, J.B. (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283-287.

APPENDIX B

Standard Operating Procedure (Courtesy Dr. K. Gordon)

Enterococcus esp **Assay Protocol for inhibitory samples**

Scope of Application

This protocol applies to the extraction of *Enterococcus* spp. DNA from colonies growing on membrane filters incubated on mEI plates from sites where there is PCR inhibition. DNA is then use to determine the presence of the esp gene at the site(s) in question

Summary

This protocol outlines the correct procedure to handle membranes containing enterococci colonies, as well as how to perform DNA extraction and PCR amplification of the esp gene.

Apparatus/Supplies

- Qiagen DNeasy Blood and Tissue Kit and ASL buffer (purchased separately, Cat #s 69506 and 19082 resp) or
- Qiamp DNA Stool mini kit (not as cost effective as above option)
- InhibitEX tablets (Qiagen, Cat # 19590)
- Ice cold 100% Ethanol (VWR Cat# EMD-4450)
- 15ml or 50ml centrifuge tube (VWR Cat# 89039-666 and 89039-658)
- 2X GoTaq® Green Master Mix (VWR Cat# PAM7122 Or PAM7123)
- 10 mM esp forward and esp reverse primer
- Nuclease free/ PCR grade water- comes with GoTaq mix or can be purchased separately (Fisher Sci cat # bp2484-50)
- 0.2 ml Thin walled PCR tubes (USA Sci Cat# 1402-8100)
- 0.5 ml Tube (USA Sci Cat# 1605-0099)
- DNA away (VWR cat# 53509-506)
- Thermocycler
- mEI plates
- 0.45 µm nitrocellulose filter
- Azide Dextrose broth (VWR cat# 90003-102)

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation with through latex gloves and with inhalation of fumes.

Procedures

When handling filters, it is easy to cross-contaminate samples with the forceps. When possible, a new pair of forceps should be used for each sample. Each pair should be decontaminated by dipping them into reagent alcohol and flaming then dipping into or spraying ends with DNAaway. The DNAaway must then be wiped off with a paper towel. The forceps can then be flamed again prior to use or for added precautions the forceps can be cross-linked in the cross linker, twice at an energy setting of 600.0 (~2 minutes each) before being used to transfer filters. It is especially important that this procedure be followed while transferring the filters from the mEI plates to the azide dextrose tubes.

The spike and method blank (MB) help keep track of cross-contamination therefore, the MB should always be processed after the spike. The spike should be processed either as the very first 'sample' or after all the samples and before the MB. Samples should be treated in the same order for the whole procedure.

I. Sample Processing (McQuaig *et al.* 2006)

- Filter 300ml of each sample through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

II. Controls

- Prepare two positive controls, each of which will be spiked with 100µl of a 10^{-5} dilution of an overnight culture of *Enterococcus faecium* C68, which contains the esp gene. These controls are termed spike 1 (SP1) and spike 2 (SP2). SP1 is a 300 ml composite sample of each of the sites sampled while SP2 is 300 ml of buffered dilution water.
- Prepare a method blank (MB) consisting of 300ml buffered dilution water filtered through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

III. Enrichment Step (McQuaig *et al.* 2006)

Have ready 15 mL screw-cap tubes (1 per sample), each containing 5 ml azide dextrose broth (Difco) [Azide Dextrose broth= 34.7g/L water]. Lift filters containing enterococci colonies from mEI plates with sterile tweezers which have been treated with DNAaway, crumble/fold with the aid of another pair of forceps or a sterile swab, and place into the top of the tube. Push the filter down with a sterile swab. Vortex vigorously and incubate for 3 hours at 41^{o}C with vigorous shaking to wash bacteria from the filters and enrich the culture.

IMPORTANT NOTE: CHANGE GLOVES FOLLOWING ANY VISIBLE CONTACT WITH LIQUID!! USE BARRIER PIPET TIPS THROUGHOUT!

IV. Preparation for Extraction (with Qiagen DNeasy Blood and Tissue Kit and ASL or the Qiamp DNA Stool mini kit)

- Spray bench with (1) 70% ethanol and wipe, and (2) DNA Away and wipe
- Pre-heat the heating block to 95°C and ensure the 70°C is on and at 70°C.
- Set out and label 1 filter spin column per sample and 1 for extraction control.
- Label 3 sets of 2 ml microcentrifuge tubes for (a) initial centrifugation, ASL and addition of InhibitEX tablet (b) Proteinase K and then transfer of inhibitEX supernatant and (c) final DNA elution step. These tubes should be crosslinked twice for two minutes while open in a microcentrifuge tube tray. The tubes should then be closed while still in the crosslinker.
- You will need one extra tube in each set for the extraction blank.
- Aliquot reagents into 15 ml or 50 ml sterile, screwcap tubes (ASL, AL, ethanol, proteinase K, AW1, AW2, AE) or microcentrifuge tube (proteinase K).

V. Extraction for non-inhibitory sites (Modified from Manufacturers instructions)

- From each sample/control, pipet 1.8 ml into a 2 ml microcentrifuge tube. Excess culture can be stored at 4ºC in case of problems with extraction.
- Centrifuge culture tubes at high speed in microcentrifuge^{c} 2-3 min. to pellet.
- Decant the supernatant and resuspend the pellet in 1.2 ml ASL lysis buffer (Qiagen, Inc.).
- Vortex for 1 min or until homogenized. Also set up the extraction blank (ASL buffer only). Transfer tubes to heating block and incubate at 95ºC for 5 min.
- Add 1 InhibitEX tablet (Qiagen, Inc.) to each sample and vortex immediately and continuously for 1 min until tablet is completely suspended. Incubate for 1 min at room temperature to allow inhibitors to absorb to the InhibitEX.

- Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX.
- Pipet 200 µl of supernatant (be sure not to get any of the pellet) into a new microcentrifuge tube and add 15 µl of Proteinase K (Qiagen, Inc.). Vortex.
- Add 200 µl Buffer AL (Qiagen, Inc.) and vortex for 15 s.
- Transfer tubes to 70°C incubator for 10 minutes.
- Add 200 µl ice cold absolute ethanol and vortex the samples immediately.
- Transfer the resulting suspension to filter spin columns, followed by centrifugation at 10,000 × g for 1 min. Remove tubes from centrifuge SLOWLY to avoid wetting the column (this caveat applies to next steps). If the column does get wet just re-centrifuge the sample(s). Discard the collection tube.
- Place columns into new collection tubes and wash each column with 500 µl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. Discard collection tube.
- Place columns into new collection tubes and wash each column with 500 µl **buffer AW2** (Qiagen, Inc.) by centrifugation at 14,000 × g for 3 min. VERY CAREFULLY remove from centrifuge and ensure none of the filtrate has splashed back on the column. It is crucial that the column is dry before starting the next step. Discard collection tube.
- Place each column in its CORRESPONDINGLY LABELED MICROCENTRIFUGE TUBE. Add 200 µl buffer AE (Qiagen, Inc.) to the column. Let buffer sit in column 5 min. before centrifugation to maximize yield. Elute DNA by centrifugation at 10,000 × g for 1 min. Store the eluate at -20ºC until used as PCR template.

VI. PCR

- Add template to tubes on bench (NOT under hood) that has been cleaned with DNA Away. Always run a no-DNA PCR negative control in addition to a positive control reaction with *Enterococcus faecium* C68 DNA as the template.

Recipe per reaction using GoTaq Green Mix (Promega; Taq, dNTPs, buffer and gel loading dye included)

- 25 µl GoTaq Green Mix
- 15 µl H_2O
- 2.5 µl *esp* forward primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-TAT GAA AGC AAC AGC ACA AGT T-3'(Scott *et al.* 2005)
- 2.5 µl *esp* reverse primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-ACG TCG AAA GTT CGA TTT CC-3'-(Hammerum and Jensen 2002)
- 5 µl template

PCR Cycle:

- Initial 94°C for 2 min.

30 cycles of:

- 94°C 1 min
- 58°C 1 min
- 72°C 1 min

1 cycle of

- Final 72°C for 5 min

Hold at 4°C

VII. Electrophoresis

- Have ready a 2.0% agarose gel
- Ethidium bromide to stain the DNA can either be added directly to the gel (1ul of 1% EtBR per 50ml of gel) or to the running buffer (20ul of 1% EtBR added to 1L 1X TAE). 1% EtBr solution is a 1%w/v solution in water (eg. 0.1g in 10ml water).
- Load the Promega 100 bp ladder in the first lane (6ul per lane).
- Load 10ul of sample into each lane .
- Run the gel at 90 V for ~ 45 min.
- The expected product is 680 bp.

References

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McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R. and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Appl Environ Microbiol* **72**, 7567-7574.

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APPENDIX C MicroLog™ Microbial Identification System (MIS) Data

Isolate name	ID result	ID #1 name
28A-5	Species ID	*Enterococcus faecium*
28A-6	Genus ID : *Enterococcus*	*Enterococcus mundtii*
28A-8	Species ID	*Enterococcus casseliflavus*
28A-9	Genus ID : *Enterococcus*	*Enterococcus mundtii*
28A-10	Genus ID : *Pediococcus*	*Pediococcus acidilactici/parvulus*
28C-1	species ID	*Enterococcus faecium*
28C-6	species ID	*Enterococcus mundtii*
28C-7	genus ID : *Enterococcus*	*Enterococcus mundtii*
28C-9	genus ID : *Enterococcus*	*Enterococcus faecalis*
28C-10	genus ID : *Enterococcus*	*Enterococcus hirae*
28C-11	species ID	*Streptococcus criceti*
29C-3	species ID	*Enterococcus faecium*
29C-5	species ID	*Enterococcus faecium*
29C-11	species ID	*Enterococcus faecium*
29C-12	species ID	*Enterococcus mundtii*
29C-15	species ID	*Pediococcus acidilactici/parvulus*
29C-17	species ID	*Enterococcus hirae*
29C-18	species ID	*Enterococcus faecium*
31B-2	species ID	*Enterococcus faecium*
31B-3	species ID	*Enterococcus gallinarum*
31B-5	species ID	*Enterococcus faecium*
31B-6	species ID	*Streptococcus hyointestinalis*
31B-11	species ID	*Enterococcus dispar*
31B-12	species ID	*Enterococcus faecium*
31B-14	species ID	*Alloiococcus otitis*
31B-16	species ID	*Enterococcus faecium*
31B-17	genus ID : *Pediococcus*	*Pediococcus acidilactici/parvulus*
31B-19	species ID	*Enterococcus faecalis*
32C-12	species ID	*Enterococcus casseliflavus*
32C-14	species ID	*Enterococcus casseliflavus*
32C-17	genus ID : *Enterococcus*	*Enterococcus mundtii*
32C-25	species ID	*Enterococcus solitarius*
32C-28	species ID	*Enterococcus casseliflavus*

33C-9	genus ID : *Enterococcus*	*Enterococcus faecium*
33C-10	species ID	*Enterococcus faecalis*
33C-12	species ID	*Pediococcus pentosaceus*
33C-13	species ID	*Enterococcus faecium*
33C-14	species ID	*Pediococcus acidilactici/parvulus*
33C-15	species ID	*Pediococcus acidilactici/parvulus*
33C-16	genus ID : *Enterococcus*	*Enterococcus gallinarum*
33C-18	species ID	*Alloiococcus otitis*
33C-20	genus ID : *Enterococcus*	*Enterococcus faecalis*
33C-23	species ID	*Enterococcus casseliflavus*
35A-1	species ID	*Enterococcus faecium*
35A-2	species ID	*Pediococcus pentosaceus*
35C-6	species ID	*Enterococcus casseliflavus*
35C-7	species ID	*Enterococcus gallinarum*

APPENDIX D Evironmental Conditions Data

Date	24 h rainfall (cm)	7 d rainfall (cm)	Sample site	Water Temp (°C)	Salinity (ppt)	pH	Turbidity (ntu)	Dissolved oxygen (mg l^{-1})
2/17/10	0.0	2.3	NUE028	12	27.0	8.2	1.5	9.8
			NUE029	12	27.0	8.2	1.5	9.8
			NUE031	12.4	26.9	8.2	1.6	10.1
			NUE032	12.4	26.9	8.2	1.6	10.1
			NUE033	12.4	26.9	8.2	1.6	10.1
			NUE035	12.4	26.9	8.2	1.6	10.1
3/24/10	0.0	1.8	NUE028	18.8	26.2	7.9	14.1	4.4
			NUE029	18.8	26.6	8.0	12.5	4.5
			NUE031	18.8	26.9	7.9	15.8	4.4
			NUE032	18.8	27.0	77.0	10.8	5.1
			NUE033	18.7	27.0	8.1	9.4	4.4
			NUE035	18.8	27.0	8.0	12.2	4.1
4/28/10	0.0	0.0	NUE028	21.7	30.1	6.5	20.5	4.8
			NUE029	21.7	30.0	7.4	19.3	5.1
			NUE031	21.8	30.1	7.6	6.6	5.7
			NUE032	21.8	30.1	7.7	8.6	4.8
			NUE033	21.8	30.2	7.7	9.4	4.8
			NUE035	22.1	30.1	7.9	14.1	5.2
5/16/10	2.5	2.5	NUE028	27.9	29.5	7.9	17.2	NA
			NUE029	27.5	29.7	7.9	20.3	NA
			NUE031	28.5	30.0	7.9	11.4	NA
			NUE032	27.9	30.0	7.9	15.2	NA
			NUE033	28.0	29.2	7.8	12.4	NA
			NUE035	30.6	30.0	7.9	19.4	NA
5/26/10	0.0	0.0	NUE028	26.5	33.4	8.1	13.6	7.7
			NUE029	26.6	33.5	8.1	12.7	7.6
			NUE031	27.0	33.3	8.1	19.7	7.2
			NUE032	26.9	33.4	8.1	19.6	7.6
			NUE033	27.3	32.4	8.1	12.5	8.4
			NUE035	27.6	32.0	8.1	11.0	6.9

Date	24 h rainfall (cm)	7 d rainfall (cm)	Sample site	Water Temp (°C)	Salinity (ppt)	pH	Turbidity (ntu)	Dissolved oxygen (mg l^{-1})
6/3/10	0.0	3.6	NUE028	28.4	30.4	7.9	14.2	NA
			NUE029	28.1	30.4	8.0	11.1	NA
			NUE031	28.8	30.8	8.0	30.6	NA
			NUE032	30.1	30.8	8.0	33.9	NA
			NUE033	29.4	31.2	8.1	19.9	NA
			NUE035	29.8	31.1	8.0	8.0	NA
6/19/10	3.6	4.3	NUE028	29.2	32.0	8.0	29.9	6.3
			NUE029	29.4	32.0	8.0	31.1	6.5
			NUE031	29.6	31.4	8.0	48.5	6.1
			NUE032	29.6	31.4	8.0	48.5	6.1
			NUE033	28.3	31.1	7.8	na	6.1
			NUE035	29.3	31.1	8.0	25.8	6.5
7/6/10	0.0	5.8	NUE028	30.0	32.8	8.1	24.4	7.2
			NUE029	30.4	32.8	8.1	22.9	7.7
			NUE031	30.4	33.0	8.1	27.1	7.3
			NUE032	30.3	32.8	8.1	9.8	7.3
			NUE033	30.5	32.5	8.0	11.1	7.8
			NUE035	31.4	32.2	8.1	13.3	7.8
9/10/10	0.0	9.1	NUE028	29.0	33.7	8.0	19.3	5.7
			NUE029	29.0	33.7	8.0	25.5	5.6
			NUE031	28.9	33.4	8.0	30.7	5.8
			NUE032	29.4	33.5	8.0	29.0	5.7
			NUE033	29.5	33.0	7.9	22.3	5.0
			NUE035	29.4	33.3	8.0	24.0	6.0
9/22/10	.25	21.0	NUE028	27.0	26.8	7.9	38.3	6.3
			NUE029	27.1	26.6	8.0	50.0	6.1
			NUE031	27.1	26.4	8.0	42.4	6.8
			NUE032	27.1	26.5	8.1	41.7	6.3
			NUE033	27.1	26.2	8.0	40.3	6.3
			NUE035	27.1	26.2	8.0	26.3	6.7

Date	24 h rainfall (cm)	7 d rainfall (cm)	Sample site	Water Temp (°C)	Salinity (ppt)	pH	Dissolved Oxygen (mg l-1)
			OST18499	31.18	0.28	7.48	3.7
7/7/10			OST18500	30	0.53	7.52	5.51
			OST18501	32.03	0	7.71	5.09
			OST18499	31.3	0.88	7.88	NA
			OST18500	29.41	1.54	7.61	5.9
9/13/10	1.45	7.325	OST18501	29.55	0.26	777	3.63
			OST20559	30.72	0.75	7.2	3.96
			OST20198	32.19	0.28	7.59	NA
10/18/10			OST18499	22.1	1.42	7.51	3.73
10/18/10	0	0	OST18500	21.95	3.56	7.58	NA
10/18/10			OST18501	22.23	0.2	7.88	NA
			OST18499	14.73	1.59	7.47	4.51
			OST18500	13.96	3.32	7.72	9.45
12/6/10	0.05	0.05	OST18501	12.2	0.25	8.18	9.28
			OST20559	21.33	1.29	7.56	8.99
			OST20198	NA	NA	NA	NA
			OST18499	15.85	0.76	7.61	5.38
1/19/11	0	5.95	OST18500	14.96	0.9	7.67	8.32
			OST18501	15.31	0.18	7.91	7.73
			OST18499	21.41	1.76	7.49	3.62
			OST18500	21.19	3.5	7.68	7.28
3/9/11	0	0.075	OST18501	20.63	0.57	7.91	8.06
			OST20559	22.41	1.5	7.57	8.63
			OST20198	NA	NA	NA	NA
			OST18499	25.75	1.25	7.56	3.54
4/20/11	0	0.025	OST18500	24.76	2.8	7.61	5.87
			OST18501	24.43	0.64	7.89	6.91