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Microbial Source Tracking: Tools for Refining Total Maximum Daily Load Assessments (2005)

Stormwater Academy

Microbial Source Tracking: Tools for Refining Total Maximum Daily Load Assessments

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I. Executive Summary

For over 100 years, indicator bacteria of fecal origin have been used to assess water quality and alert managers to increased risk of the presence of human pathogens. While these indicator organisms, including total coliforms and fecal coliforms, have helped protect public health for decades, they are now being utilized as surrogates for pathogens in total maximum daily load (TMDL) programs that are mandated by the US Environmental Protection Agency. The goal of TMDL assessment, which is carried out by the Florida Department of Environmental Protection in Florida, is ultimately to identify the sources of contaminant loading to Florida waters that are listed as "impaired", and to determine how the loading can be reduced in order to return each water body to its designated use.

Many of Florida's impaired waters are listed due to high concentrations of fecal indicator bacteria (total coliforms and/or fecal coliforms). Simply measuring the concentration of these organisms provides no information about their source, as they may originate from a variety of warm-blooded, or even cold-blooded animal feces. Mounting evidence indicates that some of these indicator bacteria are capable of long-term survival and/or slow growth outside of their host's gastrointestinal tract. Although there are major questions about the continued use of indicator bacteria for water quality monitoring in the long term, State and Federal regulations and programs mandate their use for the foreseeable future.

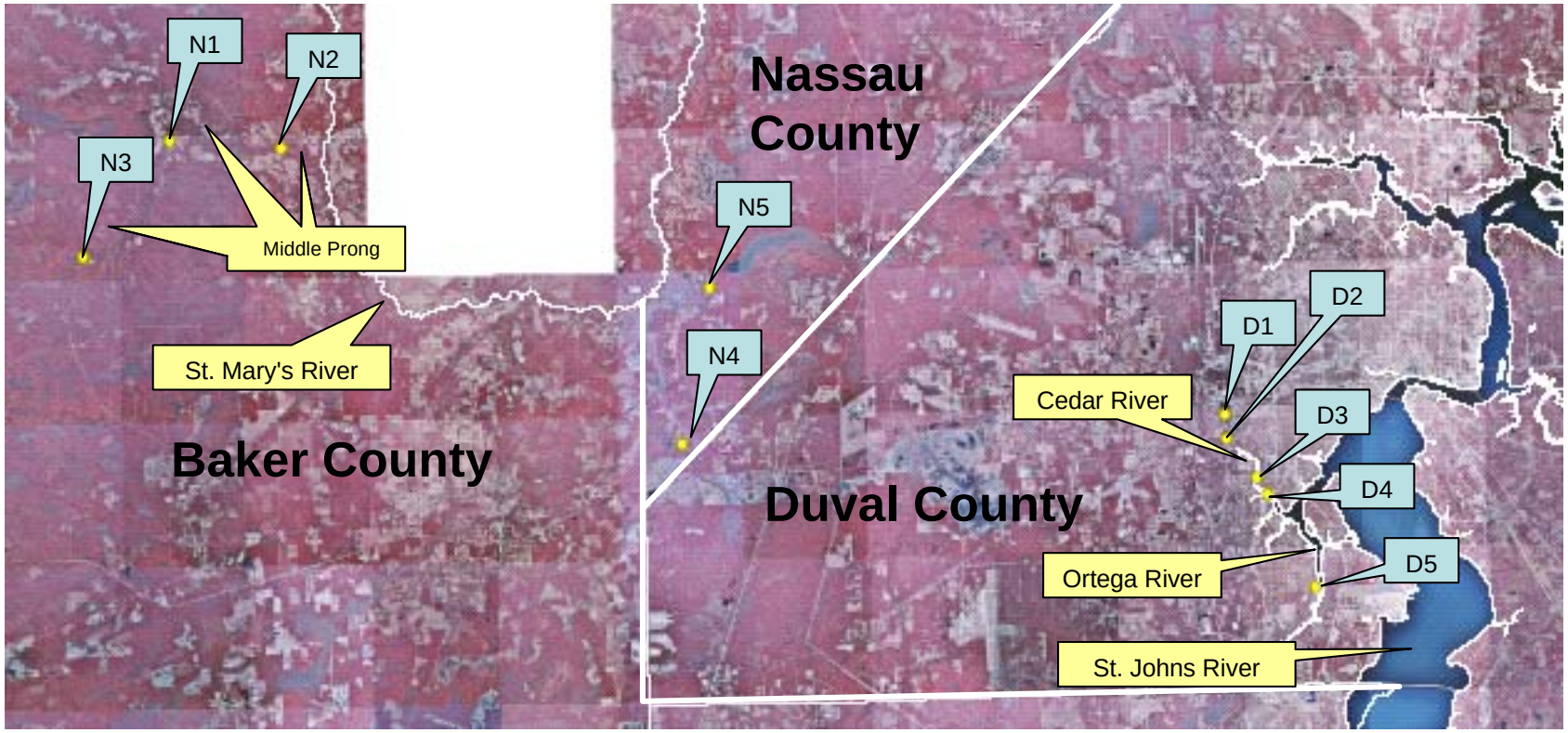
Microbial source tracking (MST) is a term that includes a "toolbox" of methodologies that are designed to determine the dominant source(s) of fecal pollution to environmental waters. Knowledge of fecal source is essential to TMDL assessment, and is also crucial for refining best management practices (BMPs) for various land uses. Furthermore, because certain types of fecal material, particularly that originating from humans, poses a relatively great health risk, knowledge of the source of fecal loading from a given watershed can help refine risk assessment for better prediction of human health risk posed by water used for recreational and shellfish-harvesting purposes.

This project was designed to test the usefulness of recently developed MST methods for determining the dominant sources of indicator bacteria in two Florida counties which represent contrasting land use: Nassau County is largely rural, and the most probable sources of impacts to

water quality include poorly functioning septic systems, livestock (mainly cattle), stormwater runoff and bacterial resuspension from sediments. The Duval County sites were located in urban Jacksonville on the Cedar River, and the most probable sources of fecal contamination were poorly functioning septic systems and aging central sewer infrastructure, wild birds, dogs and bacterial resuspension from sediments. One of the sample sites was positioned at a creek that was the site of a major sewage spill several months before the study started. The MST methods utilized were (1) creation of a library of genotypic fingerprints of *Enterococcus* spp., (2) detection of the human-associated *esp* gene of *Ent. faecium* by PCR, (3) detection of a human-associated *Bacteroides* strain by PCR for the 16S rRNA gene, and (4) detection of a ruminant-associated *Bacteroides* strain by PCR for the 16S rRNA gene. Because of the expense of building the library, the library-dependent method (BOX-PCR) was utilized only in Duval County. An inter-laboratory study was conducted by the University of South Florida (USF) and Biological Consulting Services of North Florida (BCS). The USF and BCS laboratories carried out the MST methodologies separately to assess the inter-laboratory variability of the methods.

Indicator bacteria concentrations (fecal coliforms, *Escherichia coli* and enterococci) were measured in the water column and sediments at all sites. Bacteriological water quality levels at the Nassau County sites differed significantly (few exceedances of Florida State standards) from those in Duval County (majority of samples exceeded standards) (Executive Summary Fig. 1; see Fig. 1 in Methods section for GPS coordinates for all sites). MST analyses suggested the presence of human fecal contamination at all sites except for N3 (Nassau County), at the headwaters of the St. Mary's River. The human-associated *esp* gene was detected most frequently at Duval site D1 (five times), followed by D2 and N1 (three times each), and was most frequently detected in urban (Duval) waters. The human-associated *Bacteroides* marker was detected most frequently at D1 and D2 (four times each). The ruminant-specific *Bacteroides* marker was detected infrequently in both watersheds.

Executive Summary Figure 1. Map of sampling locations.



Genetic "fingerprints", or patterns, can be generated by many methods. In this study BOX-PCR was used. BOX refers to a group of highly conserved repeating intergenic regions in Gram-positive bacterial chromosomes (Martin et al 1992). The USF *Enterococcus* library of BOX-PCR fingerprints represented four major source categories: human, sediments, dogs and wild animals. The library was decloned (sister clones removed) before analysis so that identical fingerprints from individual fecal samples could not match themselves in the library. Jackknife analysis of the decloned library better simulates the process of matching isolates from unknown sources to the library than if sister clones are left in the library, and showed that the library was moderately predictive of fecal source. The correct classification for human source isolates was 55.6%, that of sediment isolates was 57.1%, that of dog isolates was 36.0%, and that of wild bird isolates was 50.4%. For the BCS library the correct classification for human source isolates was 46.0%, that of sediment isolates was 52.8%, that of dog isolates was 41.9% and that of wildlife isolates was 59.4%. A proficiency test was performed using enterococci isolated from fecal samples collected in Duval County, Florida that were not isolated from samples included in the library. Human and sediment proficiency isolates classified well, at 66.7% and 52.0%, respectively; however dog and wild bird isolates classified poorly, at 22.2% and 26.1% in the USF library. In the BCS library, human, dog and wildlife proficiency isolates classified well, at 80.0%, 66.7% and 92.3%, respectively; however sediment isolates classified poorly, at 33.3%.

Enterococci isolated from water samples collected at Duval County sites were classified into the source categories given above using the USF BOX-PCR library. The majority of isolates at all sites were assigned to the Human and Sediment categories. The sites with the highest percentage of isolates assigned to the Human category were D1 and D2, corroborating the *esp* and *Bacteroides* results.

Human-associated genetic markers and *Enterococcus* strains were detected frequently in the urban Cedar River in Duval County. Detection was most frequent at the upstream sites, where tidal flushing and water flow are much lower than at the downstream sites. The status of wastewater treatment systems at homes along the river is not currently known, but could be investigated to determine whether failing central sewer infrastructure or old/failing septic systems may be contributing fecal contamination. The infrequent identification of human or cattle sources at the Nassau County sites suggests that other sources, such as stormwater, are

responsible for loading of indicator bacteria in these waters. Bacteria carried in runoff can be deposited in sediments, where they can survive for long periods. Differential survival and/or possible slow growth was suggested by the finding that Sediment forms a coherent source category in the BOX-PCR library. Many of the enterococci isolated from the Cedar River were assigned to the Sediment category, demonstrating the importance of bacterial resuspension in increasing microbial loads in the water column. The potential for pathogen survival in the sediment is not well understood; however, it is unlikely that most pathogens survive as long as the (apparently) sediment-adapted *Enterococcus* strains. This sediment survival phenomenon has also been noted for *E. coli*, and it poses serious drawbacks for the use of these organisms as indicators of human health risk in recreational waters (Anderson et al 2005, Davies et al 1995, Fish et al 1995, Sherer et al 1992).

This study indicates that MST methods have promise in determining the sources of fecal pollution and fecal indicator bacteria in Florida waters. The highly technical nature of the PCR-based methods requires substantial familiarization time when laboratories adopt these methodologies, and attention must be paid to standardization steps which will facilitate inter-laboratory data comparisons. The multiple test, weight-of-evidence approach toward source tracking will be required in the foreseeable future, as existing MST methods are refined and new methods are developed. A decision tree based on the results of these studies is currently under development by the participants in this study in collaboration with FDEP personnel.

II. Introduction, Objectives and Study Design

Microorganisms from fecal sources can enter waters used by the public for drinking water, recreation, or shellfish-harvesting, which poses a health threat. Microbial fecal indicator organisms have been used for over a century to warn of the risk of fecal contamination in water and food. While indicator organisms are generally not pathogenic themselves, their role in water quality monitoring is to provide the indication of increased risk of human pathogens. Indicator organisms such as *Escherichia coli* and *Enterococcus* spp. are found in the gastrointestinal tract of humans, as well as warm-blooded and some cold-blooded animals (Harwood et al 1999; Scott et al 2002). These organisms, along with enteric pathogens (which include viruses, bacteria, and protozoa), are shed in fecal material that can contaminate surface waters. Although low numbers of these enteric bacteria may be sporadically isolated from invertebrate animals, the fact that invertebrate populations are high in sediments of unimpacted waters while the number of indicator bacteria are low strongly suggests that the invertebrates make a small or undetectable contribution to indicator bacteria concentrations in impacted water bodies.

Enumerating indicator organisms in a water body cannot differentiate among the various contamination sources that may impact a given watershed (Harwood et al 2000; Simpson et al 2002; Scott et al 2005). Identification of the specific sources of the indicators would allow cost-effective remediation of contaminated waters and more accurate risk assessments, since human fecal contamination (e.g., sewage) is much more likely to contain human pathogens than fecal material from other sources. Microbial source tracking (MST) seeks to link specific microorganisms to various sources of fecal pollution. MST methods can be divided into two major groups, library and nonlibrary-based. Nonlibrary-based methods generally rely upon polymerase chain reaction (PCR) to amplify putative host-specific genes, also known as markers, present in certain microorganisms. Results are obtained on a presence-absence basis, rather than a quantitative assessment of the impact of a source on a water body. Library-based methods require the generation of large databases (libraries) of patterns (“fingerprints” or “barcodes”) generated by subtyping pure cultures of indicator organisms isolated from various fecal sources. Feces of host animals that may contribute to fecal pollution in a given watershed form the basis of the library. Indicator organisms are subsequently isolated from water samples and their

fingerprints are compared to the library of known sources to identify the probable source(s) of the contamination.

The fecal anaerobe genus *Bacteroides* has been used as a target for a nonlibrary, PCR-based source identification (Bernhard and Field, 2000). Primer sets for human-specific and ruminant- (i.e. cattle) specific strains exist and have been used successfully in field (Bernhard et al 2003) studies on the West coast. These primers have not been validated in other geographical areas. *Enterococcus faecium* is one of the dominant enterococci found in human feces, and a virulence gene (*esp*) found in this species is associated with human sewage (Scott et al 2005). A PCR assay developed for this human-specific marker has been used successfully for detecting human fecal pollution in Florida.

Libraries generated for MST analysis are usually based on one particular indicator organism, most commonly *E. coli* or *Enterococcus* spp. due to their status as recognized indicator organisms and their ubiquitous distribution in feces. Fingerprints of these organisms can be derived from phenotypic methods, such as antibiotic resistance patterns or carbon source utilization, or from genotypic methods that analyze some component of the genetic structure. A recent study comparing phenotypic MST methods demonstrated that *Enterococcus* spp. generally displayed greater predictive capability for identifying fecal source than *E. coli* (Harwood et al 2003). This was also concluded by Harwood et al. (2000) when comparing libraries composed of either *E. coli* or *Enterococcus* antibiotic resistance patterns. One study of libraries composed of ribotype patterns found that *E. coli* libraries had lower predictive accuracy for host source than enterococci libraries (Dontchev et al 2003, Amuso et al 2005). Some genotypic methods used for fingerprinting are pulsed-field gel electrophoresis, ribotyping, and repetitive extragenic palindromic-PCR (rep-PCR) (Carson et al 2003; Hahm et al 2003; Malathum et al 1998; Parveen et al 1999). To date, peer-reviewed publications regarding the efficacy of rep-PCR as a genotyping method for creating MST libraries have used only *E. coli.*

Library-dependent MST methods require a large database composed of the fingerprints of many bacterial isolates. The number of isolates included in the library is crucial to its success, as small libraries demonstrate random clustering that is independent of fecal source (Harwood et al 2003; Whitlock et al 2002). Furthermore, the diversity of indicator organism subtypes in the feces of

various animals is high (Anderson et al 2003); therefore, large libraries are required to represent the diversity of indicator organisms that may enter a water body. Genotyping methods such as ribotyping are very expensive on a per-isolate basis, while MST methods based on phenotypic patterns such as antibiotic resistance or carbon source utilization are much less expensive on a per-isolate basis. Thus, libraries based on phenotypic fingerprints may offer the possibility of regional libraries with a larger temporal scale, but the inherent sources of error that affect phenotypic observations can complicate the use of these methods.

One genotyping method that is relatively inexpensive compared to ribotyping is rep-PCR, which generates fingerprints by amplifying repetitive DNA elements present in bacterial genomes. Two repetitive elements commonly targeted are the repetitive extragenic palindromic (REP) sequence, and the A subunit of the BOX element. PCR primers are designed to read outward from the repeated DNA sequences that are located throughout the chromosome. Some studies have compared fingerprints generated by primers specific for the REP sequence and the BOX element, concluding that the percentage of correctly assigned source groups was higher when using BOX primers (Dombek et al 2000; Hassan et al 2003). Rep-PCR has primarily been used as an epidemiological tool for typing pathogen strains. Rep-PCR of *E. coli* has recently been applied to MST (Dombek et al 2000), but rep-PCR studies of enterococci have not appeared in peer-reviewed publications to date.

Objectives

Certain microbial source tracking (MST) techniques may be useful for measuring the contribution of humans and other animals to fecal indicator organism loading in Florida waters. This is particularly important since impairments of many of Florida's waters are caused by "pathogen" (fecal coliform) concentrations that exceed State standards. Total maximum daily load (TMDL) assessments and implementation rely upon accurate determinations of contamination source, which cannot be solely determined by concentrations of indicator organisms. It is generally agreed upon that none of the currently utilized methods alone are sufficient to unequivocally determine fecal source in environmental waters (Fox 2003). Therefore, a weight of evidence approach provided by several methods was used in this study by combining enumeration of indicator organisms with microbial source tracking methods to

determine the source and relative contribution of indicator bacteria in Florida waters, as well as to assess the interlaboratory variability of these techniques.

The first specific objective of the study was to determine whether contamination of surface waters by human feces could be differentiated from that of animals. The second objective involved determining whether specific MST methods could discriminate between contamination from various species. The degree of fecal contamination at the sites was determined by indicator bacteria (fecal coliform bacteria, *Escherichia coli* and *Enterococcus spp.*) concentrations at the time of sampling. Although the MST methods used in this study are not based on *E. coli* or fecal coliforms, many studies have found a strong correlation between fecal coliform concentrations and *Enterococcus* concentrations in ambient waters (McElyea 2003, Shehane et al 2005), and a recent study found that *Bacteroides* concentrations were correlated with those of *E. coli* (Dick and Field 2004)

Four MST methods (3 nonlibrary and 1 library-based) were tested during the course of this study for their ability to discriminate between human and various animal contributors. Two distinctive watersheds were investigated by the nonlibrary (presence-absence) methods: (1) the Little St. Mary's River in rural Baker/Nassau County, which was believed to be minimally impaired by anthropogenic sources, and (2) the Cedar River in Jacksonville, which is highly impaired by anthropogenic activities. The library-based method was only used for the Cedar River watershed.

Specific Goals

- Assess the contribution of human and cattle feces to fecal contamination of surface waters in rural Baker/Nassau County and in the lower Cedar River in urban Jacksonville using PCR for human-specific and ruminant-specific *Bacteroides*, and PCR for human-specific *Enterococcus*.
- Assess the contribution of dogs, humans, wild bird, and sediments to fecal contamination of the lower Cedar River *via* development of a library of *Enterococcus* rep-PCR patterns isolated from the above sources. Fingerprints of *Enterococcus* spp. isolated from tributary water samples were subsequently compared to those in the library in order to predict the dominant source(s) of enterococci.
- Assess the inter-laboratory variability associated with the MST methods.

- Assess the accuracy of several MST methods toward source ID for intentionally contaminated water samples.

Study Design

The St. Mary's River and Cedar River watersheds are characterized by contrasting land use and were tested by standard microbiological assays for fecal contamination (fecal coliform, *E. coli* and *Enterococcus*). Furthermore, the source of fecal contamination was investigated by MST methods. The watershed of the St. Mary's River in Nassau County represented relatively rural sites, while the Cedar River in Jacksonville represented an urban watershed. The latter is probably influenced by aging septic systems and central sewer infrastructure, as well as stormwater runoff. Human enteric viruses were isolated from Cedar River waters in a study conducted in 2003-2004 (Harwood, 2004), demonstrating contamination by human fecal material. Due to the expense involved in building a library, one library was constructed for the urban Cedar River watershed at USF, as well as at BCS. Two laboratories were utilized during this study so that interlaboratory agreement of molecular results could be assessed.

III. Methods

Sampling Locations and Physical/Chemical Water Quality Data. Five sampling locations were chosen in each watershed, the Cedar River and St. Marys River (Figure 1). The Cedar River watershed represents an anthropogenically impaired watershed, while the St. Marys River watershed is relatively unimpaired. Site D1 is located in a residential area and was characterized by low flow rates during the study. D2 is located in a wooded area off a major thoroughfare (Lenox Ave.). A public boat ramp (Lighthouse Marine), small private marina and residential area are located at D3. The Cedar Shores Apartment Complex off of Blanding Blvd. (a major roadway) is located on site D4. D5, the most downstream site in Duval County, was located at a JEA lift station on 118th Avenue near the Southwest Wastewater Treatment Facility. A major break in the sewer line had occurred at this site prior to the study and was repaired during the course of the study.

Sites N1-N3 are located in Baker County in the Osceola Wildlife Management Area and are surrounded by a large silviculture area. N4 and N5 are located in a slightly urbanized area of Nassau County. N3 is located at the headwaters of the Middle Prong of the St. Marys River on County Road 250 with sites N1 and N2 being downstream along County Roads 125 and 127, respectively. Several small cattle farms are located between N1 and N2 on County Road 122. N4 is located at the U.S. Highway 90 bridge crossing of the Deep Creek tributary of the St. Marys River in Nassau County. The Brandy Branch of the St. Marys is located at site N5 on County Road 121.

Figure 1. Map of sampling locations and latitude and longitude coordinates for each location:

N1: N 30° 26.940', W 082° 17.148

N2: N 30° 25.560', W 082° 13.515

N3: N 30° 22.831', W 082° 19.546

N4: N 30° 18.146', W 082° 14.949

N5: N 30° 22.630', W 082° 05.485

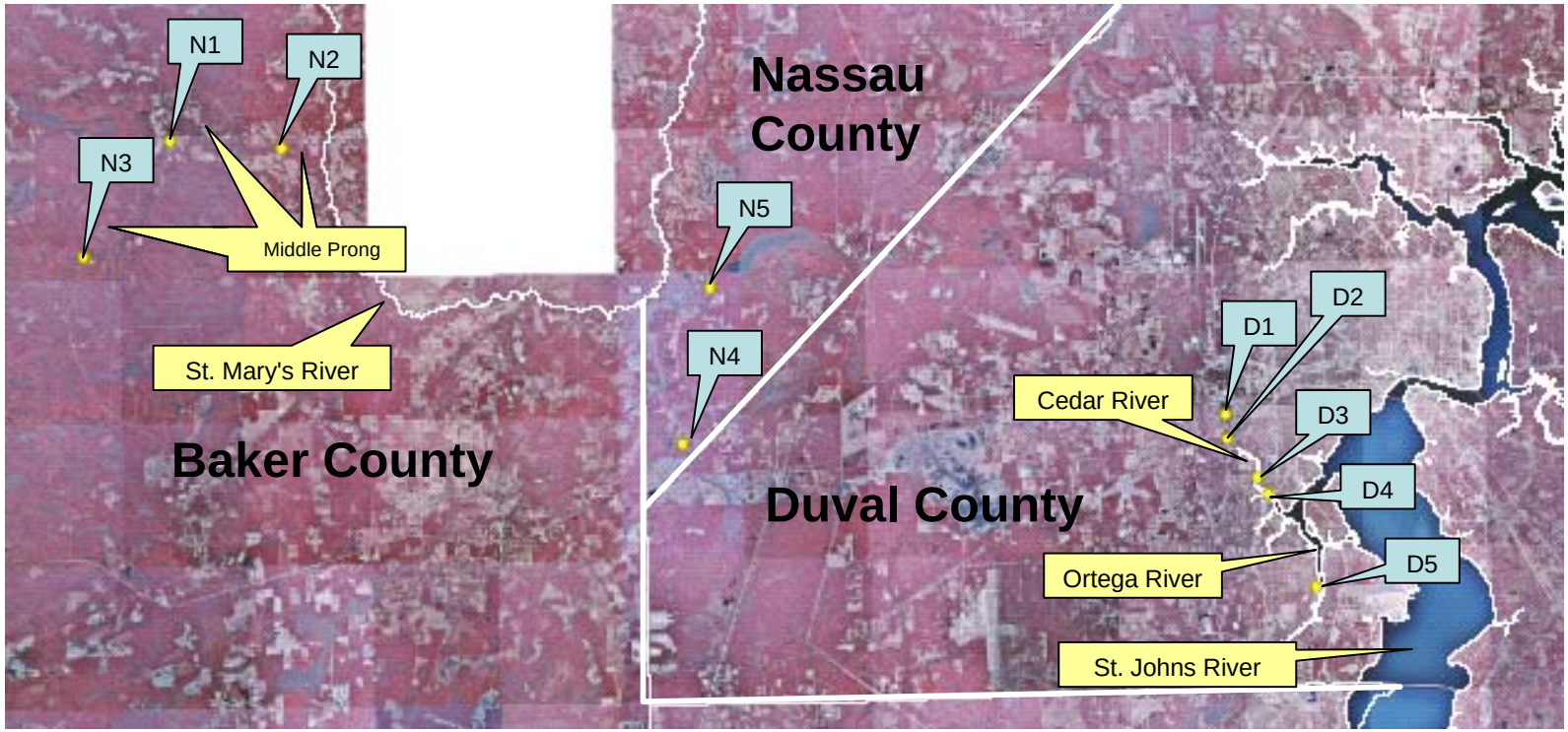
D1: N 30° 18.337', W 081° 45.212

D2: N 30° 17.550', W 081° 44.260

D3: N 30° 16.540', W 081° 44.254

D4: N 30° 16.273', W 081° 44.630

D5: N 30° 14.046', W 081° 42.424



Sample collection and handling. Water samples from sites on the Little St. Mary's River in rural Nassau County and the Cedar River in Jacksonville were collected monthly from August 2004 to May 2005 in triplicate (designated A, B, and C) from each site in sterile 1 L bottles for both USF and BCS laboratories. All triplicate samples were analyzed at both laboratories for host-associated markers, as well as BOX-PCR source typing of enterococci. The USF laboratory also analyzed each triplicate sample for indicator concentrations. Sediment samples were also collected in triplicate (designated A, B, and C) from each site using a Ponar Grab Sampler by scooping the top layer of sediment. The sediment sample (comprised of approximately 10 cm of the top sediment layer; variability between sampling locations and sampling dates was due to differences in the amount of detritus and sediment compaction) was stored in 50 mL screw-cap conical tubes until the time of processing

For development of the USF rep-PCR library fecal samples from known sources were collected from various locations throughout Duval County. Isolates for the BCS library were processed from archived samples, which originated from Duval County and various other geographical locations in the United States and Canada. Sewage was collected in 1-L sterile bottles from clarified influent at the Southwest Wastewater Treatment Facility the day prior to sampling from December through May. Sediment isolates were archived from mEI plates following sample processing from all sampling dates. Dog and wild animal source isolates were collected with sterile cotton swabs and stored in 250 µl sterile buffered water (0.0425 g L^{-1} KH_2PO4 and 0.4055 g L^{-1} $MgCl_2$) in 15 mL screw-cap conical tubes. Dog fecal samples were collected from Dogwood Park of Jacksonville one day prior to the monthly sampling event from January through May. Healthy dogs from the Duval County Animal Control Facility were sampled in December. Duck fecal material was collected from site D3 on each sampling date from December through May. Seagull feces were collected the day prior to sampling from January through May at the Little Talbot Island State Park. All samples were labeled immediately and stored on ice in a cooler within 15 min of collection.

Samples were logged on a field data sheet, which remained with the samples until they were delivered to BCS or filed at USF. A field-blank containing sterile buffered water in a screw-cap 1 L bottle was stored with the water samples for the duration of each sampling event. The field-

blank was processed in parallel with the water samples to ensure no contamination occurred during sampling and transport. All samples were processed within 12 h of collection. The USF laboratory collected fecal and sediment samples for isolation of *Enterococcus* spp. that underwent rep-PCR analysis. The rep-PCR patterns from the known sources were used in library development. Water and sediment samples were enumerated for the presence of indicator bacteria; furthermore, isolation of enterococci from water samples was performed for comparison against known sources in the library. Host-associated molecular analysis of water samples was also performed by PCR (see below). Duplicate water samples were delivered to the BCS laboratory on each sampling date.

Isolation and enumeration of indicator bacteria. Water samples were filtered through sterile nitrocellulose membranes (0.45 μm pore-size, 47 mm diameter) to enumerate fecal coliforms, *E. coli* and *Enterococcus* spp. A range of sample volumes were filtered to allow for adequate enumeration of bacterial cells. Fecal coliforms were enumerated on mFC agar (Difco) incubated for 24 h at 44.5^{o} C in a water bath (APHA, 1999). Blue colonies were counted as fecal coliforms. Enterococci were enumerated by Method 1600 (USEPA, 1996), in which filters were incubated on mEI agar (base media from Difco; indoxyl β-D glucoside from Sigma Aldrich) at 41^{o}C for 24 h. All resultant colonies with a blue halo were counted as *Enterococcus* spp. *Escherichia coli* colonies were enumerated by Method 1603 (USEPA, 2002), in which filters were incubated on Modified mTEC agar (Difco) at 35^{o}C for 2 h to resuscitate any injured or stressed cells followed by incubation at 44.5 ^{o}C for 22 h in a water bath. Red or magenta colonies were counted as *E. coli*. Plates with suitable colony numbers (10 – 50 CFU) were counted, and concentrations for were calculated by averaging the colonies from the triplicate samples. Concentrations for all indicators were then converted to CFU·100 ml^{-1} (water samples) or 100 g wet weight^{-1} (sediment samples).

A. USF MST Methods

***Bacteroides* host-associated molecular marker (non-library PCR).** Presence or absence of *Bacteroides* host-associated molecular markers essentially followed the method of Bernhard and Field (2000) at both USF and BCS. Three hundred ml of each water sample were filtered through 0.45 μm membrane filters to concentrate and collect bacteria. In case of a clogged filter, an

additional filter was utilized until a total of 300 ml were filtered. Filters were then lifted, folded carefully, and suspended in 0.5 ml guanidinium isothiocyanate (GITC) lysis solution [5M Guanidine isothiocyanate, 100 mM EDTA (pH8), 0.5% Sarkosyl] in a 1.5 mL screw-cap microcentrifuge tube. Samples were then vortexed vigorously for 1 min ensuring total immersion of the filter in the lysis solution. The filters were stored in the lysis solution overnight at -20ºC. The tubes containing the assay filters were thawed, and 0.7 ml buffer AL (Qiagen, Inc.) was added to each. The tubes were then vortexed for 30 seconds, inverted 5 times, and vortexed for an additional 30 seconds. The total volume of resulting lysate was processed for DNA extraction using a Qiagen DNeasy Tissue Kit (Qiagen, Inc.) according to manufacturer's instructions. The lysate was bound to the filter spin column by centrifugation at 10,000 × g for 1 min. The column was then washed with 500 µl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min followed by two washings with 500 µl buffer AW2 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min each. The flow through was discarded and the column was dried by centrifugation at 10,000 × g for 2 min. Purified DNA was eluted from the column with 100 µl buffer AE (Qiagen, Inc.) by centrifugation at 10,000 × g for 2 min. The eluate was stored at -20ºC until used in PCR analysis.

PCR was carried out using primers designed to amplify the human-specific marker HF8 (forward: 5'-ATC ATG AGT TCA CAT GTC CG-3', reverse: 5'-CAA TCG GAG TTC TTC GTG-3') and the ruminant-specific marker CF 151 (forward: 5'-CCA ACY TTC CCG WTA CTC-3', reverse: 5'- CAA TCG GAG TTC TTC GTG-3') (Bernhard et al., 2003). PCR reactions were performed in a 50 µL reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each of the four deoxyribonucleotides, 0.3 µM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 5 µl of template DNA. Amplification was performed with an initial step at 95 ^{o}C for 15 minutes (to activate Taq polymerase), followed by 30 cycles of 94^{o}C for 1 min, 63^{o}C (human marker) or 62 ^{o}C (ruminant marker) for 1 min, and 72^{o}C for 5 min. PCR reactions were electrophoresed on 1% agarose gels stained with ethidium bromide and visualized under UV light to assess production of PCR products of the correct size. The sizes of PCR amplicons were 525 and 580 base pairs in length for the human and ruminant-associated molecular markers, respectively. Gels were digitally documented under UV light using a

FOTO/Analyst Archiver (Fotodyne, Hartland, WI). (For amended protocol developed following the completion of the sampling dates see Appendix 1.)

***Enterococcus* human-associated molecular marker (non-library PCR).** The protocol for a human-specific PCR marker derived from *Enterococcus faecium* was developed by Dr. Troy Scott of BCS (Scott et al. 2005) at both USF and BCS. Three hundred ml of each water sample were filtered using 0.45µm membrane filters. In case of a clogged filter, an additional filter was utilized until a total of 300 ml were filtered. Filters were incubated on mEI agar (base media from Difco) at 41°C for 48 h in a water bath. Filters containing enterococci colonies were lifted, suspended in 2.0 ml Azide Dextrose Broth (Difco) in 15 mL screw-cap tubes, vortexed vigorously, and incubated for 3 hours at 41°C with shaking to wash bacteria from the filters and partially enrich the culture. Tubes were subjected to centrifugation at 7,500 × g for 10 min. The supernatant was decanted, and the resulting pellet was resuspended in 200 µl ASL lysis buffer (Qiagen, Inc.) followed by incubation at 95ºC for 5 min. DNA extraction was performed on the resulting lysate using a QIAamp DNA Stool Mini Kit according to manufacturer's instructions (Qiagen, Inc.). Briefly, 15 µl of Proteinase K (Qiagen, Inc.) was added, followed by 200 µl Buffer AL (Qiagen, Inc.). The sample was vortexed immediately and incubated at 70°C for 10 minutes. Next, 200 µl ice cold absolute ethanol was added and the sample was vortexed immediately. The resulting lysate was then transferred to filter spin columns, followed by centrifugation at 10,000 × g for 1 min. The column was washed with 500 µl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. The column was then washed with 500 µl buffer AW2 (Qiagen, Inc.) by centrifugation at 14,000 × g for 3 min. The flow through was discarded and the column was dried by centrifugation at 14,000 × g for 1 min. Purified DNA was eluted from the column with 200 µl buffer AE (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. The eluate was stored at -20ºC until use as PCR template.

Primers specific for the *esp* gene in *E. faecium* were developed by Scott et al. (2005). The forward primer, which is specific for the *E. faecium esp* gene is: (5'-TAT GAA AGC AAC AGC ACA AGT T-3'). A conserved reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3'), developed previously by Hammerum and Jensen (2002), was used for all reactions. PCR reactions were performed in a 50 µL reaction mixture containing 1 × PCR buffer, 1.5 mM

$MgCl_2$, 200 µM of each of the four deoxyribonucleotides, 0.3 µM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 5 µl of template DNA. Amplification was performed with an initial step at 95°C for 15 minutes (to activate Taq polymerase), followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were separated on a 1.0 % agarose gel stained with ethidium bromide. Gels were digitally documented under UV light using a FOTO/Analyst Archiver (Fotodyne, Hartland, WI). The expected PCR product was 680 base pairs in length.

Library-based MST: rep-PCR of *Enterococcus* spp. The library was composed of *Enterococcus* spp. isolated from feces of animals that were suspected of impacting the Cedar River watershed, i.e. dogs, ducks, seagulls, and other wild bird, and human/sewage. In addition, enterococci were isolated from Cedar River sediments due to the potential of this source acting as a reservoir of indicator bacteria. Up to 1000 isolates per source were typed from numerous fecal samples collected monthly from December 2004 to May 2005. Up to 200 enterococci isolates from sediment and sewage samples, as well as up to 20 isolates per animal fecal sample, collected from each date described above were grown in pure culture by swabbing fecal samples on mEI agar (base media from Difco) and incubated at 41°C for 24 h in order to ensure the ability to reanimate an appropriate number of isolates for further analysis. Individual colonies were confirmed as enterococci by incubation in Enterococcosel Broth (EB) (Becton, Dickinson and Co.) at 37°C for 24 h. Isolates that were positive for esculin hydrolysis (blackened media) were cryogenically preserved by adding glycerin and stored at -80°C until DNA extraction.

Genomic DNA was extracted and purified from reanimated *Enterococcus* spp. isolates by incubating thawed cells on tryptic soy agar (TSA) (Becton, Dickinson and Co.) at 37°C for 24 h. In the USF laboratory, the DNA from no more than 5 isolates per individual animal and 25 or 30 from each sediment or sewage sample, respectively, was isolated for PCR analysis in order to sufficiently represent the *Enterococcus* spp. population in each source (i.e. to reduce the likelihood of clonal isolates being analyzed). BCS isolated DNA from 23-36 isolated from six individual dogs, 23-45 isolates from two wild bird individuals, 12-45 isolates from nine duck individuals and 36 isolates from one raccoon individual (reduced the final number in the library due to a higher level of sister clones present). A single colony from each pure culture was then

inoculated into 1.75 ml brain heart infusion broth (BHI) (Becton, Dickinson and Co.) in a 2 mL, 96-deep well culture plate, and incubated at 37°C for 24 h. DNA was extracted in a 96-well format by using the Wizard SV 96 Genomic DNA Purification System (Promega) according to manufacturer's instructions. Briefly, cells were pelleted by centrifugation of the plate at 4,100 × g for 15 min, followed by removal of the supernatant media by decanting. For each well, the pellet was resuspended in 180 µl of enzymatic lysis buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, and 20 mg/ml lysozyme added just prior to use] and incubated at 55°C for 1 hr in a heating block. Each of the following were added in sequence, followed by incubations at room temperature for 2 min with shaking: 5 µl Rnase (100mg/ml) (Promega), 25 µl Proteinase K (Qiagen), 40 µl FastBreak Cell Lysis Reagent (Promega), and 250 µl Wizard SV Lysis Buffer (Promega). Lysates were then bound to a Wizard SV 96-well DNA binding plate using the supplied vacuum manifold. Each well of the binding plate was then washed 3 times with 1.0 ml Wizard SV Wash Solution (Promega) using vacuum pressure to remove each wash. Purified DNA was eluted with 100 µl nuclease-free water (Promega) using vacuum pressure into a 96-deep well microtiter plate. Purified DNA was stored at -20ºC until use as PCR template.

Rep-PCR fingerprints were generated using the protocol developed by Malathum et al. (1998) using the BOXA2R primer (5'-CTA CGG CAA GGC GAC GCT GAC G- 3'). PCR reactions consisted of the following: 5 µl Gitschier Buffer (5 ×) [83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl (pH 8.8), 33.5 mM $MgCl_2$, 33.5 µM EDTA, and 150 mM ß-mercapto-ethanol], 2.5 µl 10% dimethyl sulfoxide (DMSO), 1.5 µl primer (0.6 µM), 0.4 µl 2% bovine serum albumin (BSA), 2.0 µl dNTP mixture (10 mM each), 1.0 µl (5 units/µl) Taq DNA polymerase, and 11.6 µl DNase-free ultrapure water for a total volume of 25 µl. Rep-PCR amplicons were generated using the following thermocycle profile: initial denaturation at 95°C for 7 minutes; cycle denaturation at 90°C for 30 seconds; annealing at 40°C for 1 min; elongation at 65°C for 8 minutes; and a final elongation step at 65°C for 16 minutes. PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. Gels were digitally documented under UV light using a FOTO/Analyst Archiver (Fotodyne, Hartland, WI).

Assessment of the *Enterococcus* BOX-PCR library. All library analysis was performed in the same fashion by BCS and USF. Gel images were saved as tag image files (TIF) and transferred into BioNumerics software (Applied Maths, Kortrijk, Belgium). The *Enterococcus* BOX-PCR library was decloned before analysis by both USF and BCS laboratories in the same manner. This is a process by which identical fingerprints, or clones, from any known source sample (e.g. sewage sample or individual fecal sample) are removed from the library through the 'decloning library script protocol' in Bionumerics. Leaving clones in the library allows the matching of library isolates from a given source to their "sister clones" from the same sample, which inflates the rate of correct classification for each source. Cosmopolitan isolates (i.e. patterns shared by isolates from more than one source category) were not removed during the decloning process. Next, the library was subjected to a cluster analysis, creating a dendrogram, or grouping, of the fingerprints. The clustering technique used was "complete linkage", where a fingerprint is placed into a group, or cluster, where it is the most similar to the furthest member of that group (BCS used the UPGMA (Unweighted Paired Groups Method with Arithmetic Mean) clustering technique). These groups are the source categories human, wild, dog and sediment. Their rate of correct classification was determined by jackknife analysis using curve-based algorithms (Pearson Correlation Coefficient), which sequentially removes each isolate's pattern (fingerprint) from the library before it compares the pattern to the library to predict its source, then replaces the pattern before resuming the analysis of the next isolate (BCS used the Cosine algorithm). The comparison is based on the maximum or highest similarity between an individual pattern and a pattern(s) existing in a group.

Proficiency isolates were also used to determine the predictive ability of the library. Known source isolates (human, wild, dog and sediment) not included in the library were compared to the library by bootstrap analysis, providing a percent similarity and a confidence level for each isolate and the source category to which it was assigned. For the "human" category, enterococci fingerprints derived from sewage collected in Duval County were used as proficiencies. Proficiencies for the "sediment", "dog" and "wild bird" categories were from samples collected in Duval County (at locations where library fecal samples were collected), but withheld from the library for the bootstrap analysis.

Source identification of unknown isolates. Enterococci were isolated from the water samples (up to 100 isolates per sampling event) collected during each sampling event and archived for BOX-PCR analysis. The BOX-PCR fingerprint of each isolate was determined for those isolates that were reanimated (equally distributed throughout the study period), and each was compared to those in the library by bootstrap analysis. The percent similarity of an unknown to a source category was ≥ 90%. Results were expressed as the percentage and number of isolates from each site that were assigned to each category with a confidence level of ≥ 0.75, and including all levels of confidence as determined by ID bootstrap. The isolates that were not identified to the ≥ 0.75 confidence level were assigned to an 'unknown' category.

B. BCS MST Methods (listed only where methods differed between laboratories)

Preparation of *Bacteroidetes* template DNA for PCR reactions. PCR reactions were performed on composite DNA samples extracted from membrane filters. Water samples were filtered and filters were lifted, suspended in Qiagen Stool Lysis Buffer and vortexed vigorously. The resulting lysate was processed for DNA extraction according to manufacturer's instructions (Qiagen stool DNA extraction kit).

PCR primers and reaction conditions for Human and Ruminant *Bacteroidetes* marker. Primers specific for Bacteroidetes derived from human and ruminant sources were developed by Bernhard and Field (2000). PCR reactions were performed according to methodology outlined by Bernhard and Field (2000).

IV. Results

A. Physical/Chemical Water Quality Data.

Physical and chemical water quality data were collected at the time of sampling at each site during each sampling date using the FDEP Tallahassee YSI meter (Table 1; Appendix 2 contains the data collected at each sampling date). Temperature values did not vary between sites and exhibited the expected seasonal variations. Conductivity and pH values were higher at the Cedar River sites. Dissolved oxygen values varied between sites and across watersheds.

Table 1. Ranges of select physical and chemical parameters. See Appendix 2 for a complete list of data for all sites and sampling dates.

Site	Temp. (^{o}C)	Conductivity (μS/cm)	DO (mg/L)	pH
D1	12.3-25.5	234-485	0.66-8.21	6.95-7.35
D2	12.4-25.8	32-617	1.33-7.39	6.64-7.44
D3	12.9-27.0	33-6180	2.35-8.05	6.88-7.29
D4	13.2-28.1	326-7713	3.00-8.57	6.92-7.54
D5	12.5-29.1	371-4310	2.17-7.55	6.77-7.53
N1	12.8-24.2	72-87	3.04-9.06	3.53-4.09
N2	10.4-24.7	66-86	3.30-9.73	3.63-4.06
N3	11.8-24.1	63-89	3.99-8.01	3.57-3.97
N4	12.1-23.9	77-149	0.80-4.59	5.20-6.97
N5	12.6-23.5	79-98	1.59-8.03	4.62-5.79

B. Indicator Bacteria Concentrations

The graphs below show the geometric means of concentrations of fecal coliforms, *E. coli* and *Enterococcus* spp. (enterococci), and include a line that indicates regulatory limit for fecal coliform bacteria and enterococci (geomean 200 and 35 CFU/100 ml, respectively; Table 2 contains for an explanation of the various regulatory limits referred to in the graph). These regulatory limits apply only to water samples (Figure 2), not to sediment samples (Figure 3) collected by the USF laboratory. As predicted, water column samples from the Cedar River (D; Duval) sites had significantly higher levels of indicator bacteria ($P<0.0001$) than the Nassau County sites (N) (Figure 1). The geometric means for all Cedar sites exceed regulatory standards for enterococci, while D3 and D4 exceeded the limit for fecal coliforms. Of the Nassau County sites, N4 and N5 were in exceedance and only *Enterococcus* spp. concentrations were elevated above the standard at these sites. Graphs of data for each sample event (sample date) are provided in Appendix 3. Rainfall (average from seven days prior to each sampling event; data from the National Weather Service, Jacksonville Office) were correlated to indicator concentrations in both watersheds ($P > 0.0043$) with the relationships being stronger in the Cedar River watershed (r^2 up to 0.50).

Figure 2. Geometric means of water column samples at each site compiled from August 2005 through May 2005 (n=29 for each bar).

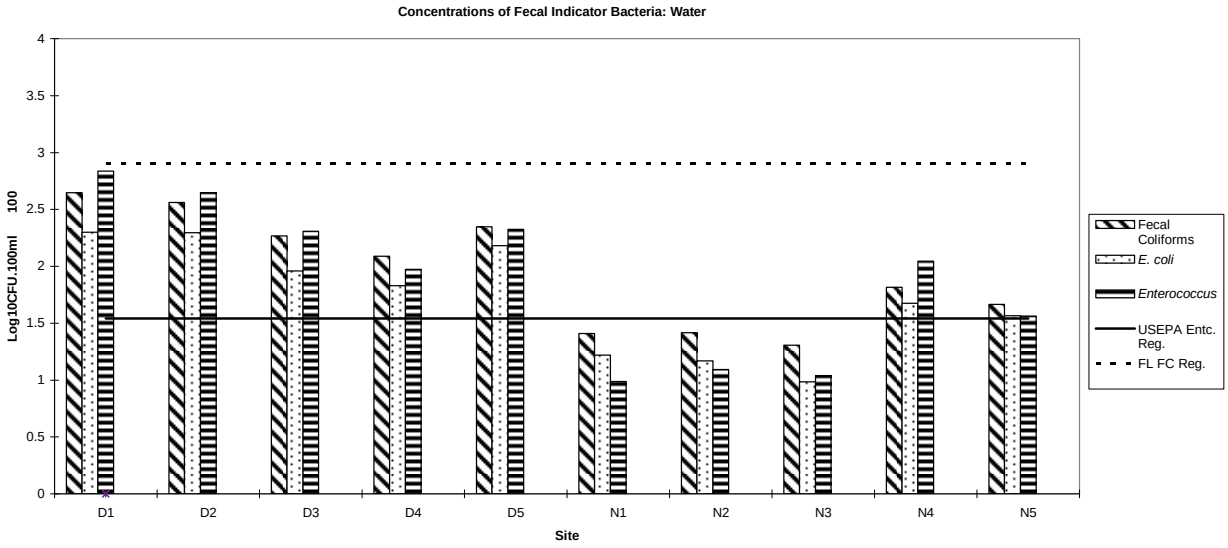


Table 2. Regulatory standards or criteria for indicator organisms in recreational waters, (CFU•100ml^{-1}).

Agency	**Fresh Water**		**Marine Water**	
	Geometric mean	One time sampling	Geometric mean	One time sampling
US Environmental Protection Agency[1]	*E. coli*- 126 enterococci- 33	*E. coli* 235-576 enterococci 62-151 (determined by illness rate and extent of body/water contact)	enterococci- 35	enterococci 104-501 (determined by illness rate and extent of body/water contact)
Florida Department of Environmental Protection[2]	total coliforms ≥1000 fecal coliforms>200	total coliforms ≥2400 fecal coliforms>800	total coliforms ≥1000 fecal coliforms>200	total coliforms ≥2400 fecal coliforms>800
Florida Department of Health[3] (Beaches monitoring)			enterococci ≥36	fecal coliforms ≥400 enterococci ≥105

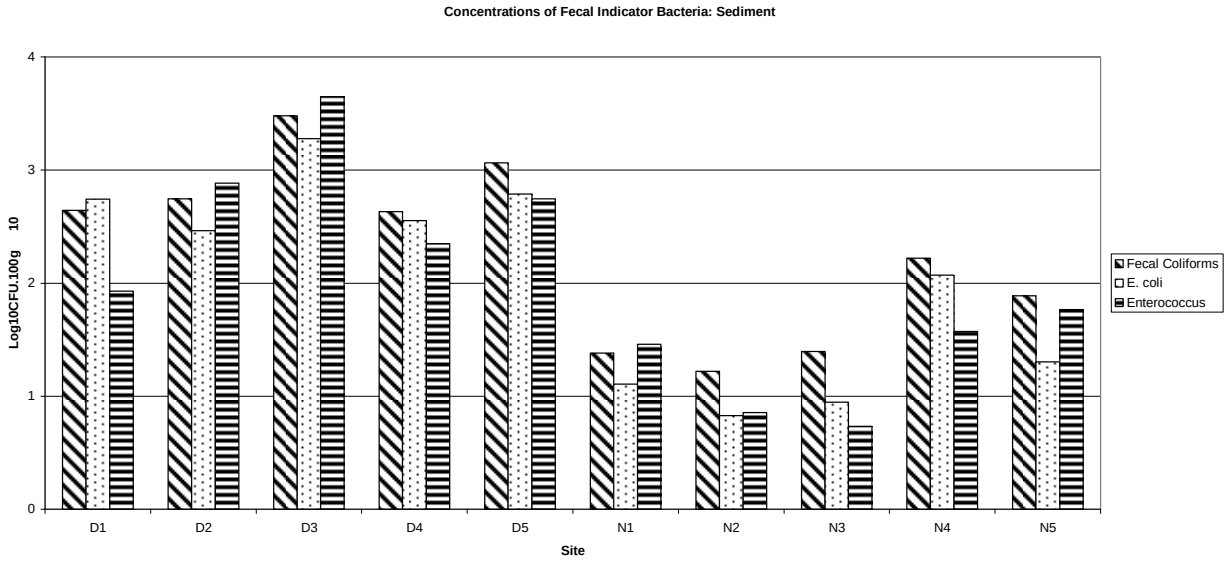
[1] US Environmental Protection Agency. 2002. Implementation Guidance for Ambient Water Quality Criteria for Bacteria. EPA-823-B-02-003.

[2] Florida Department of Environmental Protection. Criteria for Surface Water Quality Classifications. F.A.C. 62-302.530.

[3] http://esetappsdoh.doh.state.fl.us/irm00beachwater/default.aspx

Sediment samples showed differing trends that from those seen in the water column samples in Duval County (Figure 2). D3 had significantly higher concentrations of all indicator bacteria than those at the other Duval County sites. *Enterococcus* spp. concentrations were nearly tenfold higher than concentrations of fecal coliforms and *E. coli* (also noted in D1 water column samples). Bacterial counts in Nassau sediments were quite low, and sediment concentrations for each indicator were significantly higher in Cedar River sediments than in Nassau sediments ($P<0.001$).

Figure 3. Geometric means of sediment samples at each site compiled from August 2005 through May 2005 (n=29 for each bar)



A. Microbial Source Tracking

Nonlibrary-based methods for fecal source identification.

Detection of the human-specific *E. faecium esp* gene occurred more often in the impaired sites ($x^2 = 11.67$, $p = 0.001$), while the human-specific and ruminant-specific *Bacteroides* 16S rRNA gene markers were detected at similar levels in both watersheds (and $x^2 = 2.72$, $p = 0.099$ and $x^2 = 0.42$, $p = 0.838$, respectively; Table 3); for a description of validation procedures see Appendix 5 and for full list of all results for USF and BCS see Appendix 4). The markers were not detected in December and January in either watershed. Detection of the markers was not

correlated with any of the indicator bacteria concentrations in either watershed ($r^2 < 0.026$, $P < 0.058$). Data analysis for all nonlibrary MST methods is based on results from BCS due to a problem with amplicon contamination of PCR reactions in the USF laboratory during the study period that was not recognized until late in the study period for esp and a lack of amplification for the *Bacteroides* markers in environmental samples (for troubleshooting techniques used to resolve these issues see Appendix 6).

Table 3. Months with detection of *E. faecium esp* gene and human and ruminant *Bacteroides* 16S markers at each site compiled from August 2004 to May 2005 by the BCS laboratory.

Site	Human *Enterococcus* Marker	Human *Bacteroides* Marker	Ruminant *Bacteroides* Marker
D1	October, November, February, March, April	November, February, March, April	October
D2	October, February, April	October, February, March, April	ND[1]
D3	February	February	ND
D4	August, October	October	ND
D5	August, February	August	ND
N1	February, March, May	March, April	ND
N2	February	February, March, April	ND
N3	ND	ND	ND
N4	August, October	August, October	August
N5	February, March	October, February, March	October

[1]- No detection

Out of 100 sample sites/dates, the human-specific *Enterococcus esp* and *Bacteroides* primers generally agreed on the presence/absence of human fecal pollution. The *esp* gene was detected in the **absence** of human-specific *Bacteroides* in October at D1, in August at D4, and in February at D5 and N1. Conversely, human-specific *Bacteroides* were detected in the absence of *esp* in March at D2, in April at N1, and in October at N5. Therefore, the human-specific markers were in agreement at 93/100 (93%) of all sites/dates.

***Enterococcus* BOX-PCR library for fecal source identification**.

A library of BOX-PCR patterns (fingerprints) was constructed from *Enterococcus* isolates collected from four sources: 1) wild birds (or wildlife in the BCS library made by combining the duck, bird and raccoon categories), 2) dogs, 3) sewage and 4) sediment. Many more *Enterococcus* isolates were isolated from samples and typed by BOX-PCR than eventually were included in the library. Isolates processed by BCS are presented in Table 4a

Table 4. Number of enterococci isolated and typed by BOX-PCR at **USF**.

	Enterococci isolated	**Processed (rep-PCR)**	**In Decloned Library**
Human	722	721	216
Wild Bird	560	275	123
Sediment	1017	1000	287
Dog	445	340	111
Total	2744	2336	737

Table 4a. Number of enterococci typed by BOX-PCR at **BCS.**

	Enterococci isolated	**Processed (rep-PCR)**	**In Library Decloned Initially by BCS**	**In Library Decloned by USF[1]**
Human	1187	777	645	138
Dog	569	428	243	93
Raccoon	81	26	11	4
Bird	640	75	20	7
Duck	684	545	346	90
Sediment	1091	1091	1091	145
Total	4252	2942	2356	477

[1]- Isolates from outside the watershed collected by BCS comprised the following percentage of the library declined by USF: dog = 27.9%, wildlife = 51.5%, sewage = 5.8% and sediment = 0%.

Jackknife analysis (each isolate's pattern is held out of the library, compared against the library, and replaced) was used to assess the discriminatory capability of the library with respect to the four source categories (Table 5) after the library was decloned. The average rate at which isolates from the various source categories were correctly classified by at the USF lab was 49.8%, which is significantly higher than the expected rate of correct classification by chance alone (25%). The average rate at which isolates from the various source categories were correctly classified by the BCS library was 50.0% (Table 5a).

Table 5. Jacknife analysis: classification accuracy of the **USF** *Enterococcus* BOX-PCR library. Bolded cells denote percentage of isolates correctly classified into each source category.

Known Fecal Source of Isolates (n)	Predicted Source Category			
	Dog (%)	Wild Bird (%)	Human (%)	Sediment (%)
Dog (111)	**(36.04)**	(15.32)	(26.58)	(22.07)
Wild Bird (123)	(8.94)	**(50.41)**	(8.94)	(31.71)
Human (216)	(9.72)	(2.31)	**(55.56)**	(32.41)
Sediment (287)	(7.67)	(6.45)	(28.75)	**(57.14)**

Table 5a. Jacknife analysis: classification accuracy of the **BCS** *Enterococcus* BOX-PCR library. Bolded cells denote percentage of isolates correctly classified into each source category performed using USF method.

Known Fecal Source of Isolates (n)	Predicted Source Category			
	Dog (%)	Wildlife (%)	Human (%)	Sediment (%)
Dog (93)	**(41.94)**	(13.98)	(20.97)	(23.12)
Wildlife (101)	(5.94)	**(59.41)**	(13.86)	(20.79)
Human (138)	(12.41)	(7.30)	**(45.99)**	(34.31)
Sediment (145)	(10.42)	(4.17)	(32.64)	**(52.78)**

Proficiency isolates are used to determine the ability of the library to predict the source identity of an unknown isolate. Human, wild bird, sediment and dog isolates from additional fecal samples collected, but not included in the library were compared to the library (performed separately on USF and BCS libraries) by bootstrap analysis (Table 6). This provided the percent similarity of each proficiency isolate matching the source category it was assigned to, as well as the confidence level/significance for that assignment (i.e. isolates placed into a source category have at least an x% chance of being correctly identified into said category). BCS proficiency isolates included samples from bird, dog, duck, raccoon and human sources (Table 6a). The water isolates (unknowns) were compared to the library by bootstrap analysis to determine source group assignment. Table 7 (water) lists all unknowns analyzed at USF and the results for the BCS library comparison are displayed in Table 7a. All isolates were identified to a source category that had a confidence level of ≥ 0.75.

Table 6. Analysis of **USF** proficiency isolates. Human proficiencies that were correctly classified had a similarity ≥ 93.0% and a significance of 0.05 to 0.97. Sediment proficiencies that were correctly classified had a similarity ≥ 95.0% and a significance of 0.21 to 0.97. Wild bird proficiencies that were correctly classified had a similarity ≥ 92.0% and a significance of 0.05 to 0.97. Dog proficiencies that were correctly classified had a similarity ≥ 94.0% and a significance of 0.08 to 0.91.

Source Proficiency Isolates (n)	Predicted Source Category			
	Human n (%)	Sediment n (%)	Wild Bird n (%)	Dog n (%)
Human (24)	**16 (66.7)**	7 (29.2)	0 (0)	1 (4.2)
Sediment (25)	9 (36.0)	**13 (52.0)**	2 (8.0)	1 (4.0)
Wild Bird (23)	3 (13.0)	11 (47.8)	**6 (26.1)**	3 (13.0)
Dog (18)	6 (33.3)	5 (27.8)	3 (16.7)	**4 (22.2)**

Table 6a. Analysis of **BCS** proficiency isolates. Initially twenty isolates of each source category were used for proficiency analysis, but those numbers were reduced following decloning. Human proficiencies that were correctly classified had a similarity ≥ 91.0% and a significance of 0.71 to 0.98. Sediment proficiencies that were correctly classified had a similarity ≥ 91.0% and a significance of 0.18 to 0.85. Wildlife proficiencies that were correctly classified had a similarity ≥ 91.0% and a significance of 0.12 to 0.97. Dog proficiencies that were correctly classified had a similarity ≥ 97.0% and a significance of 0.69 to 0.97.

Source Proficiency Isolates (n)	Predicted Source Category			
	Dog n (%)	Wildlife n (%)	Human n (%)	Sediment n (%)
Dog (6)	**4 (66.7)**	0 (0)	1 (16.7)	1 (16.7)
Wildlife (13)	0 (0)	**12 (92.3)**	1 (7.7)	0 (0)
Human (5)	0 (0)	0 (0)	**4 (80.0)**	1 (20.0)
Sediment (6)	0 (0)	1 (16.7)	3 (50.0)	**2 (33.3)**

Table 7. Unknown water isolates were compared to the **USF** decloned library by bootstrap analysis. Similarity of each isolate to the source category was ≥ 97.1%, with significance ≥ 0.75.

Site (n)	Human n (%)	Sediment n (%)	Wild n (%)	Dog n (%)	Unidentified n (%)
D1 (184)	35 (19.0)	19 (10.3)	5 (2.7)	13 (13.1)	112 (61.1)
D2 (256)	61 (23.8)	47 (18.4)	7 (2.7)	9 (3.5)	132 (51.5)
D3 (295)	23 (7.8)	42 (14.2)	8 (2.7)	15 (5.1)	207 (70.1)
D4 (151)	10 (6.6)	7 (4.6)	4 (2.6)	2 (1.3)	128 (84.8)
D5 (76)	1 (1.3)	3 (4.0)	3 (4.0)	4 (5.2)	65 (85.5)

Table 7a. Unknown water isolates were compared to the **BCS** decloned library by bootstrap analysis using the USF method. Similarity of each isolate to the source category was ≧ 81.0%, with significance ≥ 0.75.

Site **(n)**	Human n (%)	Sediment n (%)	Wildlife n (%)	Dog n (%)	Unidentified n (%)
D1 (151)	0 (0)	0 (0)	2 (1.3)	0 (0)	149 (98.7)
D2 (145)	0 (0)	0 (0)	0 (0)	0 (0)	145 (100)
D3 (218)	0 (0)	0 (0)	2 (1.0)	1 (0.5)	215 (98.5)
D4 (206)	0 (0)	1 (0.5)	2 (1.0)	0 (0)	203 (98.5)
D5 (147)	0 (0)	0 (0)	3 (2.0)	0 (0)	144 (98.0)

V. Discussion

This research study was designed to explore the usefulness of emerging MST methods for assessment of sources of fecal indicator bacteria to Florida waters. The primary objectives of the study are listed below.

1. Determine whether these MST methods can differentiate human from animal fecal pollution.
2. Determine whether these MST methods can further discriminate among various animal sources of pollution.

The secondary objective of the study was to determine whether the methodologies were transferable between laboratories, and whether results from two different laboratories would be comparable.

The **secondary objective**, that of interlaboratory variability, was highly problematic (see Appendix 6 for troubleshooting). Although the laboratories (BCS and USF) worked closely together, and communication was constant, the time constraints of this study coupled with processing of hundreds of samples and isolates limited the time available for consultation on methodology and data analysis. At the beginning of the study, the USF laboratory had more experience with the BOX-PCR fingerprinting of enterococci, while the BCS laboratory had more experience with the *Bacteroides* human and ruminant-specific assays. The importance of familiarity of the laboratory workers with the methods became very apparent as the study progressed. Many of the planned interlaboratory comparisons of results could not be conducted because of invalid results due to factors such as contamination by DNA from PCR products in the USF laboratory, and with PCR inhibition of the *Bacteroides* PCR assays. On the other hand, the BOX-PCR fingerprints produced by the BCS laboratory were not as reproducible or high-resolution as those produced in the USF laboratory; therefore comparisons between library results in the two laboratories were difficult.

The interlaboratory comparison was not conducted in vain, as we learned a great deal about interlaboratory calibration of the highly technical PCR assays, and the results from the two laboratories combined produced a complete data set of a quality and extent that has not

previously been accomplished in a MST study. Furthermore, we learned a valuable lesson in terms of the amount of time required for a laboratory to adopt and scale up the use of these methods. We recommend at least 3 to 6 months of experience by a technican *already trained in molecular biology* with the individual methods before they are put into practice. Training of technicians should be carried out in a laboratory that is routinely performing the methods whenever possible.

The **primary objectives** of the study were more completely addressed. Objective 1 was approached through the use of host-specific genetic markers that were amplified by PCR. It should be noted that the conclusions are based on results from only one laboratory (BCS). The human-specific markers (*Bacteroides* and *esp* gene of *Enterococcus*) were in agreement at 93/100 (93%) of all sites/dates. The sites at which human impact was most frequently recorded for the human-specific markers were logical candidates, i.e. D1 and D2 in Duval County are the most upstream of the urban Cedar River sites, located where river volume is lowest and the effects of sewage impact have the lowest chance of being diluted. Waters from the presumably unimpacted headwaters of the St. Mary's River (N3) did not produce a PCR-positive signal for the human-specific assays on any sample date, lending confidence to the prediction of human vs. nonhuman source. The results indicate sporadic contamination at all sites except N3, which may well be attributable to improperly functioning onsite wastewater disposal systems (OSTDS, or septic systems) in the rural watersheds, and a combination of OSTDS and central sewer contamination in the Cedar River. Because the PCR tests are presence-absence, rather than quantitative, the fraction of indicator bacteria contributed from human vs. nonhuman sources cannot be determined. Development of a quantitative PCR (Q-PCR) assay for the source-specific markers is technologically possible, and should be explored.

Differentiation amongst various animal sources with the library-based method was not as successful. The approach to constructing and validating the *Enterococcus* BOX-PCR library included all recent advances in MST methodologies, including decloning the library and analysis of proficiency isolates. It is desirable to use more than one isolate from each fecal sample, as this practice maximizes sample collection efficiency and better reflects the diversity of microbial populations than sampling one isolate per fecal sample. However, this practice leads to the potential problem of isolation and typing of identical sister clones from the same sample.

Decloning of genotypic libraries is necessary so that a given pattern from a given fecal sample does not have a "sister clone" to match with it. Without decloning, libraries that utilize more than one isolate per fecal sample have artificially high rates of correct classification that tend to overestimate their predictive abilities. The decloned libraries of USF and BCS has very similar internal predictive capabilities, as the averages rates of correct classification were 49.8% and 50.0% respectively.

Ultimately, a useful library must be able to accurately predict the source of patterns that are from sources outside the library. Proficiency analysis is intended to test this ability, as patterns submitted for classification are not part of the library. The USF library predicted the source of proficiency isolates from human and sediment sources at rates close to the internal correct classification rates (62.7% and 52.0%, respectively), but predicted the source of dog and wild isolates quite poorly. One of the major factors included in the low rates of correct prediction for proficiency isolates in the dog and wild categories may be that the *Enterococcus* population diversity in these hosts is underrepresented. This is particularly possible in the dog category, in which misclassified isolates are spread evenly among the source categories. Bird isolates tended to misclassify as sediment isolates, suggesting the possibility that many of the sediment isolates were originally from bird sources. Proficiency isolates from BCS performed better; however, few isolates were analyzed due to the prevalence of sister clones among the proficiency isolates. Note that proficiency isolates from sediments were most poorly classified by the BCS library, which contrasts with results from the USF library.

The implementation of the library was carried out by typing enterococci from the various sample sites and using the library to predict the most probable source category. Two strategies for isolate classification were utilized: (1) all isolate classifications were reported; or (2) only isolates classified with high confidence (>75%) were reported. Classification confidence was assessed by bootstrap analysis, which is an iterative process in which one pattern (P_1) is removed from the library, the unknown (water) isolates is compared to the library and its source is predicted, P_1 is replaced in the library, P_2 is removed, and the prediction is made again. The more frequently the unknown isolate is assigned to the same source category, the more confident one can be in that assignment. One thousand iterations of this process were carried out for bootstrap analysis in this study, and isolates with bootstrap values below 75% were placed in the "Unidentified" category.

Low bootstrap values can arise from many factors, including the lack of similarity of a given isolate to any other in the library, or the similarity of a given isolate to patterns of isolates from more than one source category.

In both the USF and BCS libraries, the majority of the enterococci isolated from water were categorized as Unidentified source. This was particularly a problem for the BCS library, in which only a handful of isolates were classified with high confidence. The reason for this anomaly is unclear at the present time, although the isolates from water were typed toward the end of the study, after the BCS had become more proficient with BOX-PCR, while the library isolates were typed throughout the study. Visual examination of the fingerprints confirmed differences in resolution of the BOX-PCR patterns from beginning to the end of the study, supporting the conclusion that substantial familiarization time is necessary for successful implementation of these genotypic assays. Most of the isolates that were classified with high confidence by the USF library were placed in the Human and Sediment categories, particularly at D1 and D2. This analysis corroborated the results of the nonlibrary methods (*esp* and *Bacteroides*), which were most frequently positive at D1 and D2. The BOX-PCR library did yield a quantitative assessment of the relative importance of sources of contamination at the various sites; however, the relatively high error rate in the proficiency analysis must be considered when interpreting these results. Few of the dog and wild proficiency isolates were correctly classified by the USF library, therefore it is not surprising that few unknown (water) isolates were classified into these categories with high confidence.

The ultimate source of the sediment isolates cannot be determined from this study; however, it is hypothesized that many of these isolates are from wild animal sources such as birds, since those proficiency isolates tended to classify as sediment isolates. Almost certainly most of these isolates originated from the feces of land-dwelling, vertebrate animals, and reached the sediments via surface waters that were contaminated from inputs such as sewage, stormwater runoff, and natural fecal discharge from animals. It is hypothesized that only a minority of the fecal indicator bacteria (whether they are fecal coliforms or enterococci) that enter the water and sediments survive (remain culturable) for more than a few hours, and that environmental conditions select for “survivor strains”. The sediment isolates then become a coherent subpopulation that groups together by library-based analysis because of similar genotypic

characteristics. The question of whether elevated indicator bacteria in sediments constitute a human health threat remains to be answered, as we know less about the survival of pathogens under these conditions than we know about indicator bacteria.

The library-based methods are relatively costly. The error rates of the libraries constructed in this study may not be acceptable for regulatory purposes, but they were able to provide corroboration for the nonlibrary methods. The niche for use of library-based analysis may be in small watersheds where sources and sites are limited, rather than in large, complex watersheds. The interpretation and utility of indicator organism concentrations for gauging public health risk have become controversial as we understand more about the ecology of these organisms, and their ability to survive and perhaps replicate outside of the host. MST methods provide another piece of the puzzle about the connection between indicator bacteria, fecal contamination from various sources, and human health risk. More rapid, specific, cost-effective methods are under development for querying the microbiological safety of recreational waters, including direct measurement of pathogens; however, the quest to protect human users of our nation's waters may never have a simple answer, as the factors that influence microbial populations in and out of their hosts are incredibly complex.

VI. Conclusions

- Mean concentrations of indicator bacteria among sites in the water column and sediment were significantly different between the watersheds; this observation applied to fecal coliforms, *E. coli* and enterococci.

- MST results suggested the presence of human fecal contamination at several sites. The human-associated *esp* marker of *Enterococcus faecium* was detected at least once at all sites except N3 (at the head waters of the St. Marys River). It was most often detected at D1 (5) followed by D2 and N1 (3 each). D1 and D2 had the greatest frequency of human *Bacteroides* marker detections with four each. The ruminant *Bacteroides* marker was detected infrequently throughout both watersheds. *esp* was detected most frequently in the urban watershed, while the *Bacteroides* markers showed no differences in detection frequency between the urbanized and rural sites. The two human-specific markers markers were in agreement as to presence or absence of human fecal contamination in 93% of observations, lending confidence to their use as predictors of sewage impact.

- The *Enterococcus* BOX-PCR library of genetic fingerprints was moderately predictive of fecal source. The classification accuracy of fingerprints derived from known fecal sources was much better than would have been achieved by chance (25.0%). In the decloned USF library (sister clones from each sample removed for greater rigor), the average rate of correct classification was 49.8%.

- In the decloned BCS library, the average rate of correct classification was 49.8%.

- Proficiency isolates were obtained from samples which were not represented in the library. These isolates were classified into the correct source categories by the USF library at rates similar to that of the library isolates for Human and Sediment categories. The BCS library classified the proficiency isolates more accurately overall, but classified the isolates into the Sediment category least accurately.

- *Enterococcus* isolates from water were classified into source categories by comparison of BOX-PCR fingerprints to the library. Results are presented as all isolates classified (e.g.,

Table 6a), and as only those isolates that classified with ≥ 75% confidence in the USF library. The majority of all isolates from all water samples were assigned to the Sediment source category; however, among the isolates classified with 75% confidence, the majority was assigned to the Human category.

- Percentages of all isolates assigned to the Human category in the USF library were highest at D1 and D2 (sites with the lowest flow rates) (45.1 and 42.2%, respectively), corroborating the *esp* and human *Bacteroides* results.
- The predictive accuracy of the BOX-PCR *Enterococcus* library is far from perfect; however the successful proficiency analysis of human and sediment sources suggests that its predictive power is somewhat robust, i.e. that isolates do not have to be part of the library to be classified with moderate success. Furthermore, when the library was used in conjunction with a nonlibrary MST method (*esp*), results were complementary, strengthening the confidence in the analyses.
- It was not possible to merge the BOX-PCR libraries from BCS and USF, as interlaboratory variability was too great. Toward the end of the study the resolution of the patterns became more similar as the BCS technicians gained familiarity with BOX-PCR, suggesting that future libraries may be merged if this proves desirable.
- The finding that many of the *Enterococcus* isolates from water were assigned to the Sediment category suggests that resuspension of sediments in this area contributes greatly to the microbial load in these waters. Conversely, little is known about pathogen survival in sediments, which would be directly connected to human health risk. The correlation between pathogen and indicator survival in sediments should be investigated in Florida (and in other geographic locations) in order ensure that protection of both public health and economic interests achieved by State and Federal programs.
- The results of this study suggest that MST methods can play an important role in determining the probable sources of indicator bacteria contamination in Florida surface waters. A weight of evidence approach that combines sanitary surveys, hydrology and MST methods can inform regulatory and management decisions ranging from shellfish harvesting, to beach use, to TMDL assessments.

VII. References

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VIII. Appendices

Appendix 1.

Amended *Bacteroides* Host-Associated Molecular Marker Protocol.

Presence or absence of *Bacteroides* host-associated molecular markers followed the method of Alice C. Layton (unpublished), Center of Environmental Biotechnology, University of Tennessee (email: alayton@utk.edu). Briefly, 60-ml of a water sample was filtered through a 0.2µm syringe filter (32mm diameter) using a manual plunger or vacuum pump attached to the filter outlet for samples with high levels of silt. The filter cartridge was detached from the syringe and a sterile female luer adapter was attached to the filter outlet. A 10-ml syringe (plunger removed) was attached to the opposite end of the female luer adapter. The filter cartridge with syringe attached was rested over a 2ml microcentrifuge tube and 1.0ml of sterile 10mM Tris (pH 8.0) (Qiagen elution buffer AE can be used as well) was pipetted into the syringe barrel. The plunger was carefully replaced in the syringe barrel in order to back-flush the solution from the filter into the microcentrifuge tube. The filtrate was centrifuged at 10,000 rpm for 10 minutes to pellet cells. The supernatant (0.9ml) was removed and the pellet was resuspended in the remaining 0.1ml of supernatant. The suspension was stored at -20ºC until used in PCR analysis, in which 2.5µl served as the PCR template.

The presence of *Bacteroides* spp. was first confirmed using general primers for the genus *Bacteroides* (Bac32F: 5'-AAC GCT AGC TAC AGG CTT-3'; Bac708R: 5'-CAA TCG GAG TTC TTC GTG-3') before host-specific primers were used. PCR reactions were performed in a 50 µL reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each of the four deoxyribonucleotides, 0.3 µM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 5 µl of template DNA. Amplification was performed with an initial step at 95 ^{o}C for 15

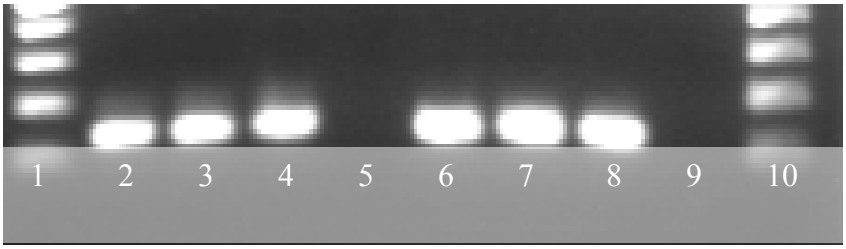
minutes (to activate Taq polymerase), followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min. The final step was an extension cycle of 10 min at 72°C. Samples were tested for inhibition of the PCR reaction by running 4 reactions per sample/assay. Three reactions are analytical replicates containing DNA from the sample only, and the fourth contains sample DNA but is also spiked with a known amount of a plasmid containing the *Bacteroides* 16S rRNA gene insert. Furthermore, concentrating a sample larger than 60-ml should be avoided in order to reduce the chance of concentrating impurities that can lead to PCR inhibition.

PCR for host-associated markers was carried out using primers designed to amplify the human-specific marker HuBac566 (forward: 5'- GGG TTT AAA GGG AGC GTA GG -3'; reverse: 5'- CTA CAC CAC GAA TTC CGC CT -3') and the ruminant-specific marker BoBac367 (forward: 5'- GAA G(G/A)C TGA ACC AGC CAA GTA -3'; reverse: 5'- GCT TAT TCA TAC GGT ACA TAC AAG -3') (Layton, unpublished). PCR reactions were performed in a 50 µL reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each of the four deoxyribonucleotides, 0.3 µM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 5 µl of template DNA. Amplification was performed with an initial step at 95 °C for 15 minutes (to activate Taq polymerase), followed by 30 cycles of 94°C for 1 min, 60°C (human marker) or 57 °C (ruminant marker) for 1 min, and 72°C for 1 min with a final extension cycle of 10 min at 72°C.

PCR reactions were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV light to assess production of PCR products of the correct size. The sizes of PCR amplicons were 676, 126 and 100 base pairs in length for the general, human and ruminant-associated molecular markers, respectively. Gels were digitally documented under UV light using a FOTO/Analyst Archiver (Fotodyne, Hartland, WI).

USF conformation of host-associated *Bacteroides* PCR primers and cross-reactivity test (Layton revised protocol)

Plasmids cloned with both human and ruminant target sequences were given to USF by Dr. Layton to be transformed into competent *E. coli* cells to maintain control DNA for the host-specific *Bacteroides* PCR assays. Plasmids were extracted from both human and ruminant transformed *E. coli* and confirmed using the aforementioned PCR protocol using host marker specific primers. Cross-reactivity was tested by attempting to amplify human marker with the ruminant-specific primers as well as ruminant marker with human-specific primers. Neither cross-reaction showed any amplification, while all plasmid extractions amplified with the appropriate primers gave robust amplification.



1. 100bp DNA ladder
2. Human-associated marker cloned plasmid #1 (human specific primers)
3. Human-associated marker cloned plasmid #2 (human specific primers)
4. Human-associated marker cloned plasmid #3 (human specific primers)
5. Human-associated marker cloned plasmid #1 (ruminant specific primers)
6. Ruminant-associated marker cloned plasmid #1 (ruminant specific primers)
7. Ruminant-associated marker cloned plasmid #2 (ruminant specific primers)
8. Ruminant-associated marker cloned plasmid #3 (ruminant specific primers)
9. Ruminant-associated marker cloned plasmid #1 (human specific primers)
10. 100bp DNA ladder

Appendix 2.

Cedar River and St. Marys River physical and chemical water quality data.

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: August 18,2004 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (C)	Salinity (ppt)	pH	Flow (m/s)/depth	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	9:00AM	25.83	0.10	7.00	-/0.5	5.09	57.40
D2	Water/Sediment	9:30AM	26.05	0.10	7.02	-/0.5	5.67	14.74
D3	Water/Sediment	8:05AM	26.83	0.58	6.97	-/0.48	4.27	17.50
D4	Water/Sediment	10: 00AM	28.20	1.34	6.99	-/0.5	5.94	87.10
D5	Water/Sediment	10:35AM	29.75	0.25	7.13	-/0.5	7.10	76.00
N1	Water/Sediment	11:45AM	24.56	0.04	6.0	-/0.5	8.13	88.20
N2	Water/Sediment	12:00PM	24.64	0.04	6.0	-/0.5	8.95	87.60
N3	Water/Sediment	12:20PM	25.23	0.04	6.0	-/0.5	9.45	88.90
N4	Water/Sediment	1:00PM	24.70	0.04	6.0	-/0.5	9.00	42.12
N5	Water/Sediment	1:15PM	24.49	0.04	6.0	-/0.5	8.61	75.25

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: September 20, 2004 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	DO (mg/L)	UV ($\mu W/cm^2$)	Depth (ft.)
D1	Water/Sediment	8:00AM	22.88	0.14	6.81	2.84	2.78	0.456
D2	Water/Sediment	8:10AM	23.06	0.14	6.67	1.60	2.33	1.579
D3	Water/Sediment	8:30AM	25.43	0.14	6.91	2.40	2.74	0.512
D4	Water/Sediment	8:50AM	25.73	0.14	6.79	2.68	2.30	0.526
D5	Water/Sediment	9:15AM	27.47	0.26	6.96	5.69	1.76	0.533
N1	Water/Sediment	10:50AM	23.31	0.02	6.67	4.13	52.40	0.578
N2	Water/Sediment	11:15AM	23.52	0.03	6.69	4.74	48.40	0.623
N3	Water/Sediment	10:30AM	23.30	0.03	6.78	2.37	26.00	0.783
N4	Water/Sediment	11:55AM	22.01	0.03	6.82	5.84	74.50	0.588
N5	Water/Sediment	12:15PM	23.32	0.03	6.81	1.02	61.20	0.473

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: October 25, 2004 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:57AM	19.78	0.20	7.03	0.765	3.57	0.63
D2	Water/Sediment	8:20AM	20.81	0.21	7.05	0.869	2.28	3.89
D3	Water/Sediment	8:35AM	22.95	0.18	7.23	2.095	3.94	15.08
D4	Water/Sediment	8:50AM	22.99	0.43	7.22	1.292	4.48	14.90
D5	Water/Sediment	9:20AM	21.53	0.18	6.91	0.925	2.84	16.97
N1	Water/Sediment	10:33AM	19.22	0.04	3.65	3.439	3.43	42.22
N2	Water/Sediment	11:15AM	19.30	0.03	3.63	2.408	7.36	95.10
N3	Water/Sediment	10:50AM	19.42	0.03	3.63	1.040	5.37	91.90
N4	Water/Sediment	12:00PM	20.04	0.04	5.66	0.804	6.67	102.00
N5	Water/Sediment	12:20PM	19.41	0.04	4.62	1.520	1.42	116.00

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: November 15, 2004 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:50AM	17.70	0.23	7.36	0.921	5.31	7.70
D2	Water/Sediment	8:25AM	18.50	0.24	7.27	1.739	3.66	9.05
D3	Water/Sediment	8:40AM	18.75	0.25	7.37	1.180	4.83	18.45
D4	Water/Sediment	8:55AM	18.61	0.34	7.44	0.902	5.92	9.12
D5	Water/Sediment	9:15AM	18.05	0.41	6.94	1.077	4.06	20.90
N1	Water/Sediment	10:35AM	14.67	0.04	3.83	5.193	5.14	82.50
N2	Water/Sediment	10:55AM	16.18	0.03	3.95	3.233	6.45	80.70
N3	Water/Sediment	11:15AM	16.51	0.03	3.95	0.838	7.32	86.80
N4	Water/Sediment	12:00PM	16.29	0.07	6.48	1.046	4.23	61.70
N5	Water/Sediment	12:20PM	15.16	0.05	4.82	1.367	1.93	71.50

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: December 13, 2004 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:50AM	12.65	0.16	7.29	1.38	8.20	2.65
D2	Water/Sediment	8:05AM	12.44	0.16	7.49	1.84	7.39	2.52
D3	Water/Sediment	8:25AM	14.69	0.13	7.42	4.50	5.28	9.45
D4	Water/Sediment	8:50AM	14.10	0.12	7.56	2.44	5.84	3.84
D5	Water/Sediment	9:10AM	13.84	0.24	7.49	18.4	5.15	14.30
N1	Water/Sediment	10:30AM	11.78	0.04	3.99	4.38	7.01	27.60
N2	Water/Sediment	10:50AM	12.77	0.03	4.08	1.86	8.33	22.90
N3	Water/Sediment	11:10AM	13.65	0.03	4.11	3.22	8.74	35.40
N4	Water/Sediment	11:55AM	12.10	0.04	7.48	2.76	8.42	38.50
N5	Water/Sediment	12:15PM	12.64	0.04	5.26	3.67	3.57	71.80

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: January 10, 2005 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	8:05AM	18.67	0.22	6.95	1.50	4.82	0.34
D2	Water/Sediment	8:23AM	19.39	0.24	6.95	3.00	3.85	0.40
D3	Water/Sediment	8:40AM	20.27	1.32	7.03	2.39	4.25	0.93
D4	Water/Sediment	9:05AM	20.41	1.52	7.07	2.62	4.46	4.87
D5	Water/Sediment	9:25AM	18.76	1.22	6.77	0.74	2.17	8.93
N1	Water/Sediment	10:40AM	16.76	0.04	3.66	2.78	4.88	7.04
N2	Water/Sediment	11:00AM	17.06	0.04	3.62	3.27	6.43	8.13
N3	Water/Sediment	11:25AM	17.00	0.04	3.60	2.92	7.25	25.6
N4	Water/Sediment	12:15PM	17.51	0.05	6.80	2.31	5.44	42.9
N5	Water/Sediment	12:35PM	17.94	0.04	5.90	3.07	3.02	34.2

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: February 08, 2005 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:50AM	12.29	0.21	7.39	0.400	7.66	4.77
D2	Water/Sediment	8:15AM	12.42	0.30	7.27	0.513	7.78	7.26
D3	Water/Sediment	8:40AM	12.97	3.40	7.30	0.505	8.12	19.37
D4	Water/Sediment	9:05AM	13.20	4.31	7.35	0.568	8.75	19.18
D5	Water/Sediment	9:30AM	12.51	2.32	7.01	2.440	7.55	37.30
N1	Water/Sediment	10:40AM	10.31	0.04	3.87	0.674	7.91	85.30
N2	Water/Sediment	11:00AM	10.68	0.04	3.93	0.520	8.95	74.10
N3	Water/Sediment	11:15AM	10.44	0.04	3.93	0.510	9.73	78.00
N4	Water/Sediment	12:00PM	10.88	0.06	6.51	0.553	8.57	89.50
N5	Water/Sediment	12:20PM	11.11	0.05	5.21	0.539	4.64	110.10

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: March 14, 2005 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:40AM	17.01	0.22	6.84	0.367	6.12	9.10
D2	Water/Sediment	8:05AM	17.47	0.23	6.99	0.135	5.59	9.22
D3	Water/Sediment	8:25AM	17.79	0.63	7.28	0.945	7.91	6.14
D4	Water/Sediment	8:55AM	17.91	1.01	7.33	0.512	7.31	23.2
D5	Water/Sediment	9:20AM	17.70	0.85	6.76	0.100	1.30	22.1
N1	Water/Sediment	10:30AM	16.29	0.04	3.65	0.501	5.52	14.18
N2	Water/Sediment	10:50AM	16.26	0.04	3.66	1.520	7.14	56.60
N3	Water/Sediment	11:05AM	15.81	0.04	3.57	0.704	8.54	26.50
N4	Water/Sediment	11:50AM	17.59	0.06	6.77	0.496	5.80	22.8
N5	Water/Sediment	12:10PM	17.29	0.04	4.85	0.501	3.19	45.2

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: April 18, 2005 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:40AM	14.26	0.18	7.25	0.932	7.75	2.14
D2	Water/Sediment	8:00AM	16.66	0.18	7.13	0.504	6.79	7.19
D3	Water/Sediment	8:20AM	18.57	0.16	7.30	0.512	5.97	23.60
D4	Water/Sediment	8:45AM	19.37	0.49	7.18	0.509	6.48	32.40
D5	Water/Sediment	9:00AM	17.26	0.20	6.82	1.280	3.46	13.85
N1	Water/Sediment	10:12AM	14.56	0.03	3.81	.0556	5.62	53.80
N2	Water/Sediment	10:36AM	14.79	0.03	3.82	0.552	7.06	73.30
N3	Water/Sediment	10:56AM	15.28	0.03	3.74	0.522	7.42	126.50
N4	Water/Sediment	11:37AM	13.42	0.04	5.75	1.135	7.33	134.70
N5	Water/Sediment	11:55AM	14.65	0.03	5.05	0.537	3.77	159.60

Field Data Sheet: Water Quality Microbiology/Microbial Ecology Lab., USF Dept. of Biology

Project Name: FDEP/MST

Sampling Date: May 09, 2005 Personnel: SDS, RU, MD

Sampling Site	Sample Type	Time	Temp. (°C)	Salinity (ppt)	pH	Depth (ft.)	DO (mg/L)	UV ($\mu W/cm^2$)
D1	Water/Sediment	7:43AM	20.36	0.21	7.20	0.105	5.35	8.32
D2	Water/Sediment	7:58AM	20.94	0.20	7.22	0.185	6.79	7.23
D3	Water/Sediment	8:14AM	22.94	1.40	7.12	1.595	8.63	26.30
D4	Water/Sediment	8:35AM	23.04	2.06	7.13	1.544	4.78	33.80
D5	Water/Sediment	8:53AM	20.95	0.55	6.73	0.180	3.17	11.74
N1	Water/Sediment	10:10AM	18.12	0.03	3.86	3.351	6.29	37.40
N2	Water/Sediment	10:30AM	18.57	0.03	3.79	0.596	7.20	74.80
N3	Water/Sediment	10:47AM	18.59	0.03	3.63	0.423	7.71	123.50
N4	Water/Sediment	11:28AM	19.50	0.05	6.22	0.484	6.19	154.50
N5	Water/Sediment	11:50AM	17.70	0.04	4.97	0.648	4.14	137.60

Appendix 3.

Indicator bacteria concentrations for water column and sediment for each sampling event.

Figure A1. Indicator bacteria concentrations in the water column at the sample sites 8-18-04.

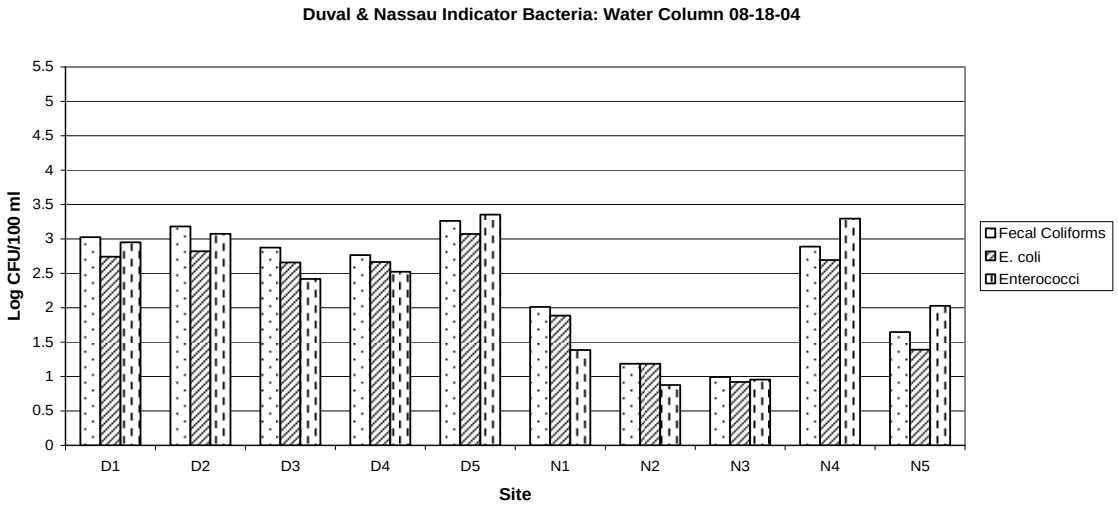


Figure A2. Indicator bacteria concentrations in sediments at the sample sites 8-18-04.

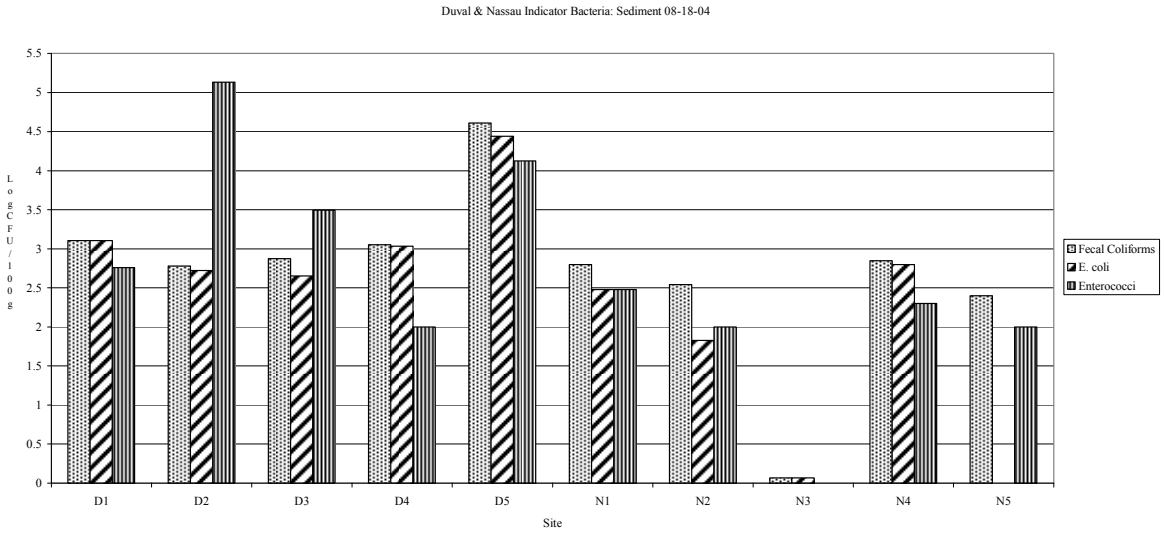


Figure A3. Indicator bacteria concentrations in the water column at the sample sites 9-20-04.

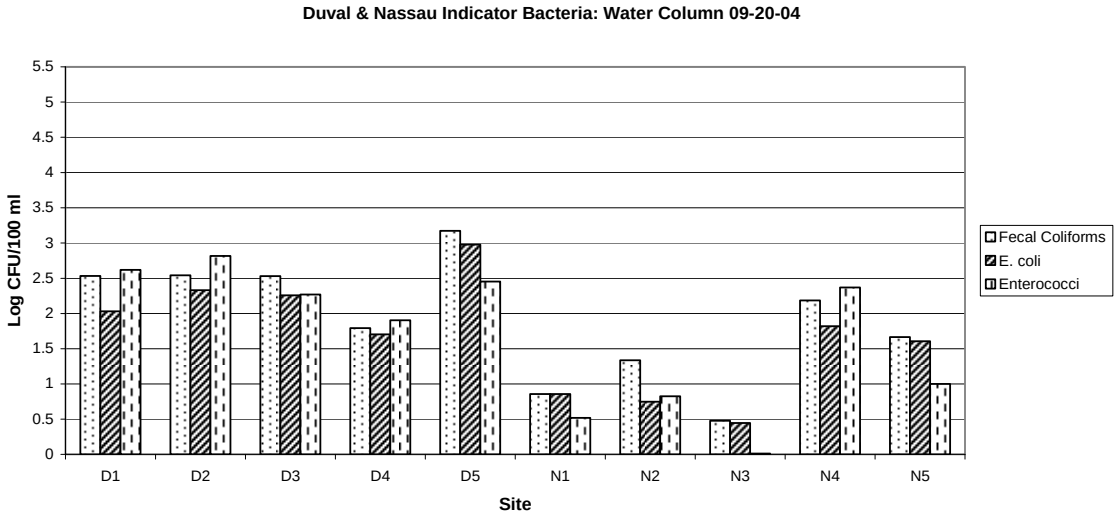


Figure A4. Indicator bacteria concentrations in sediments at the sample sites 9-20-04.

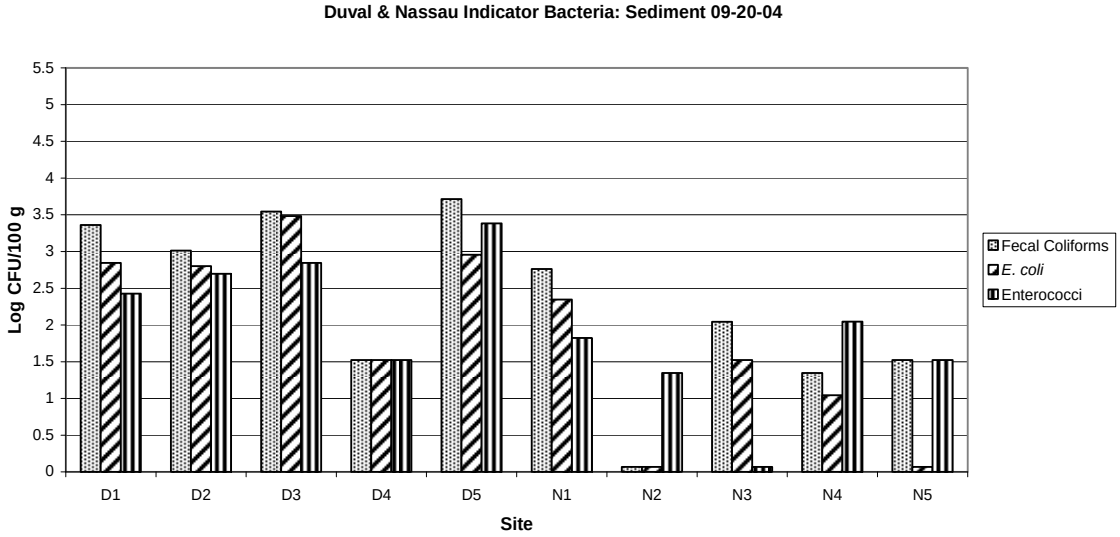


Figure A5. Indicator bacteria concentrations in the water column at the sample sites 10-25-04.

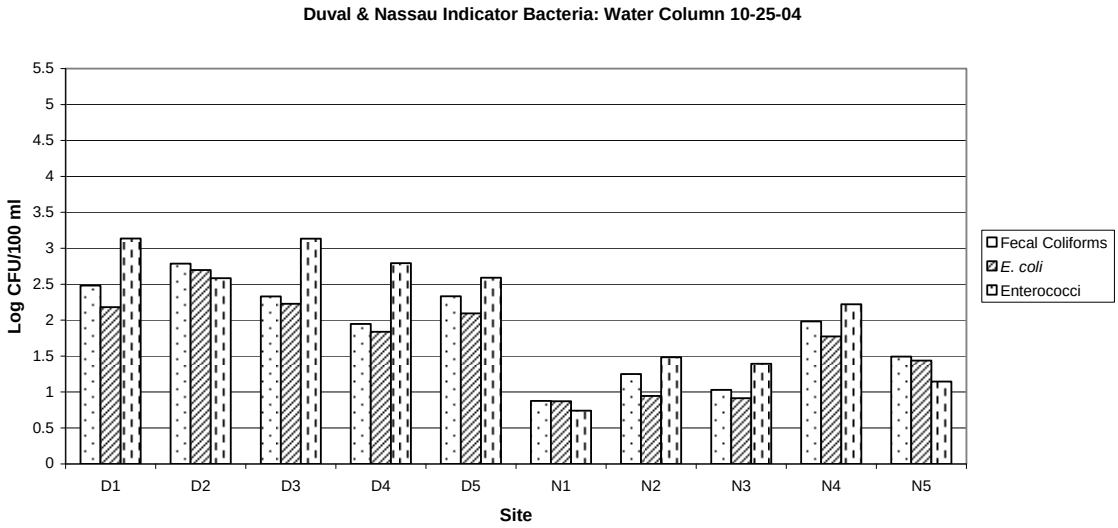


Figure A6. Indicator bacteria concentrations in sediments at the sample sites 10-25-04.

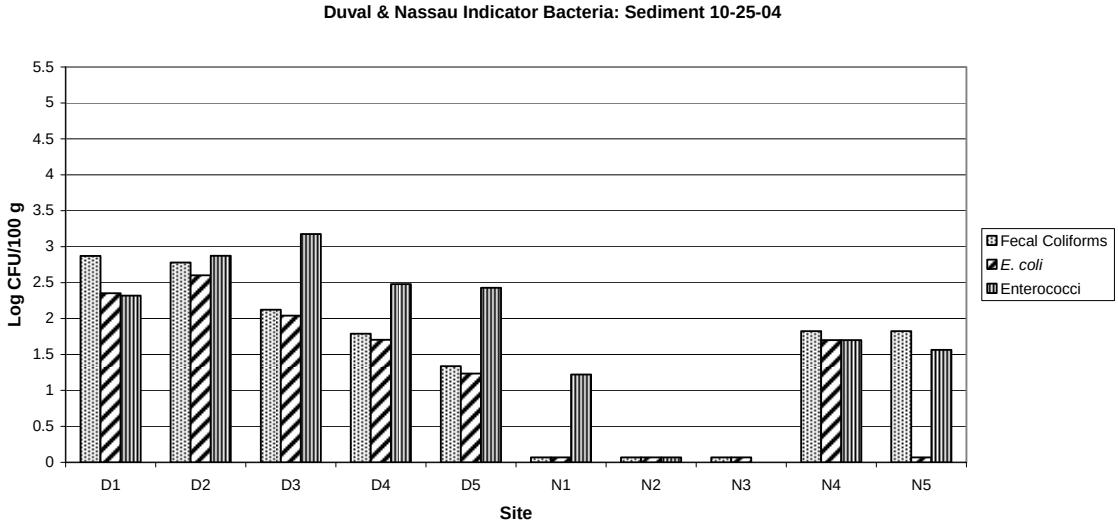


Figure A7. Indicator bacteria concentrations in the water column at the sample sites 11-15-04.

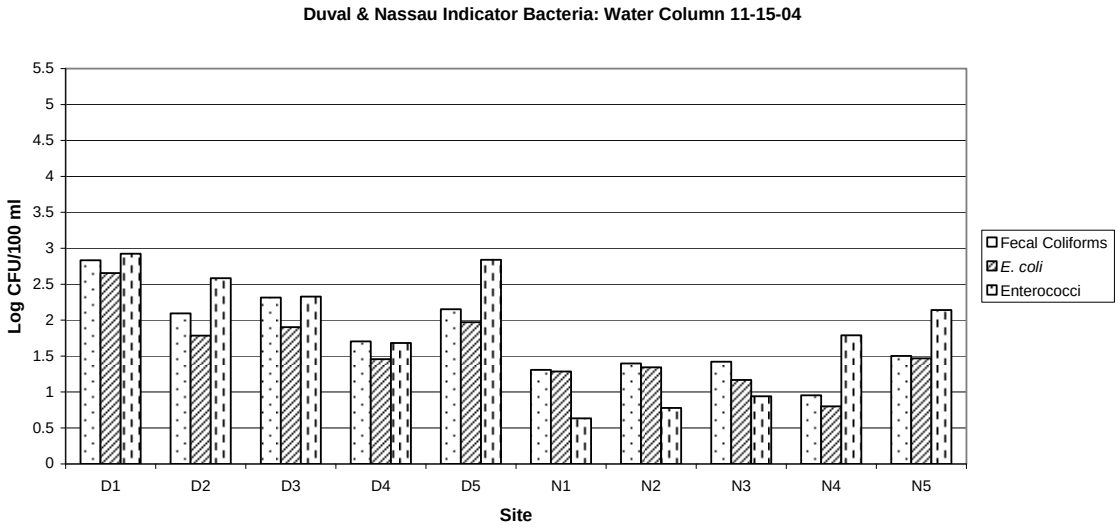


Figure A8. Indicator bacteria concentrations in sediments at the sample sites 11-15-04.

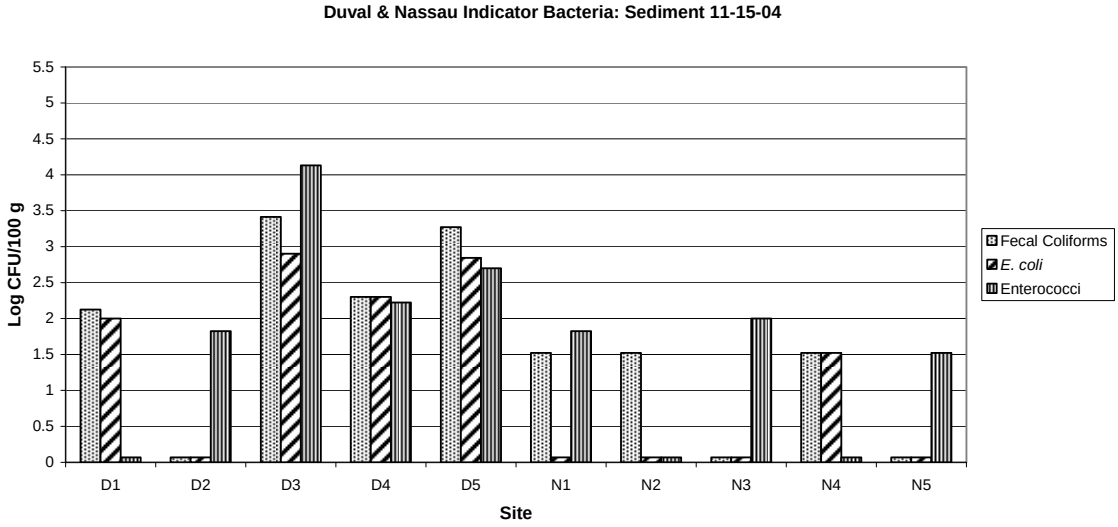


Figure A9. Indicator bacteria concentrations in the water column at the sample sites 12-13-04.

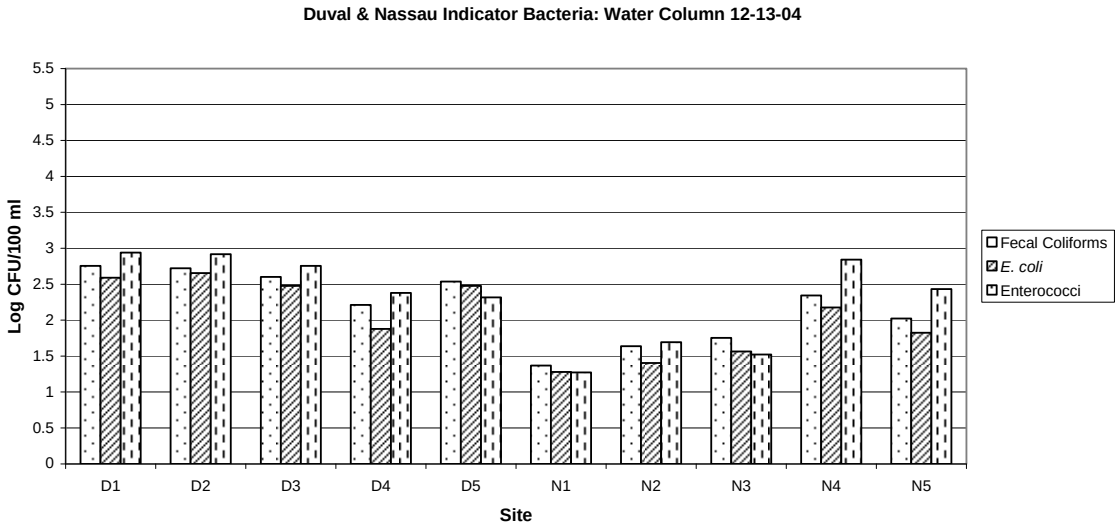


Figure A10. Indicator bacteria concentrations in sediments at the sample sites 12-13-04.

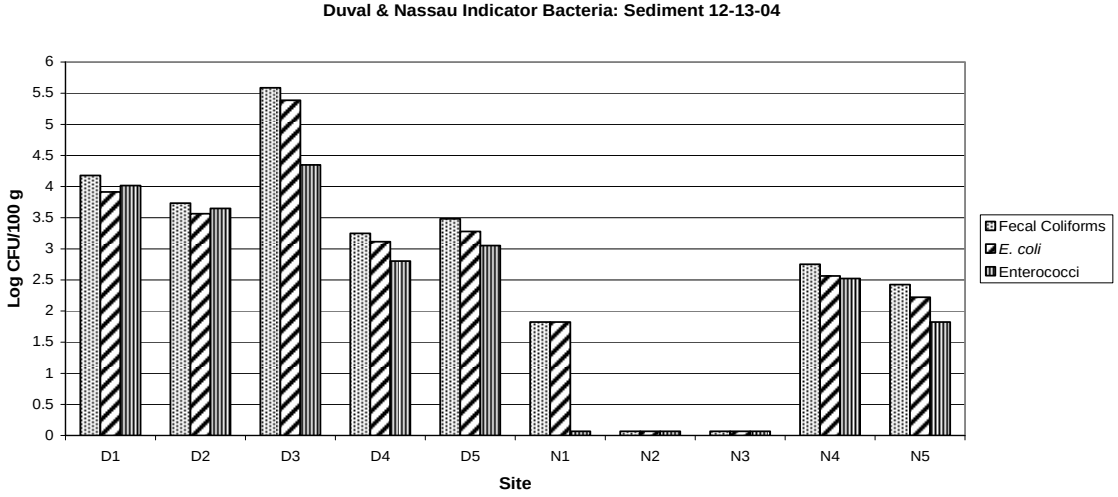


Figure A11. Indicator bacteria concentrations in the water column at the sample sites 1-10-05.

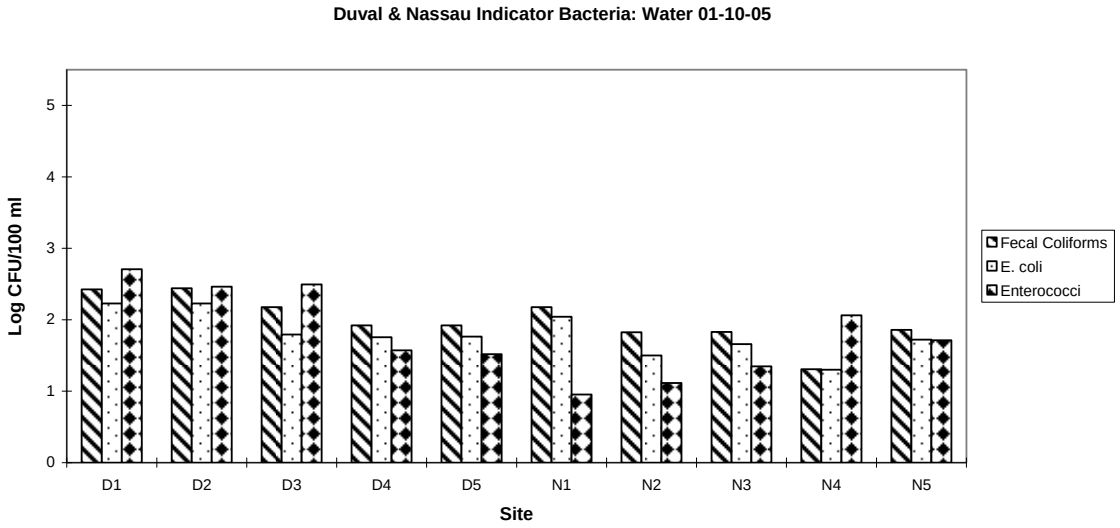


Figure A12. Indicator bacteria concentrations in sediments at the sample sites 1-10-05.

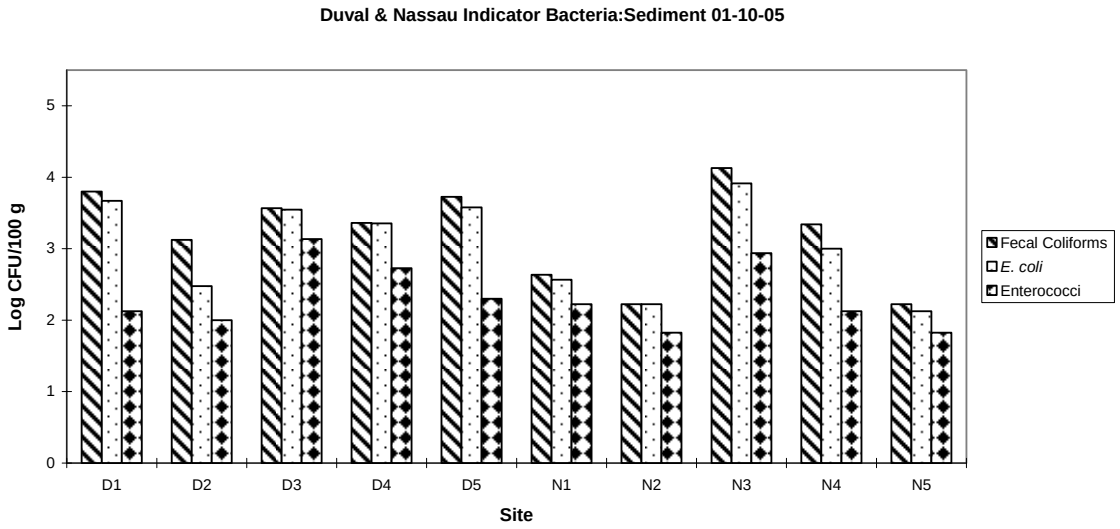


Figure A13. Indicator bacteria concentrations in the water column at the sample sites 2-09-05.

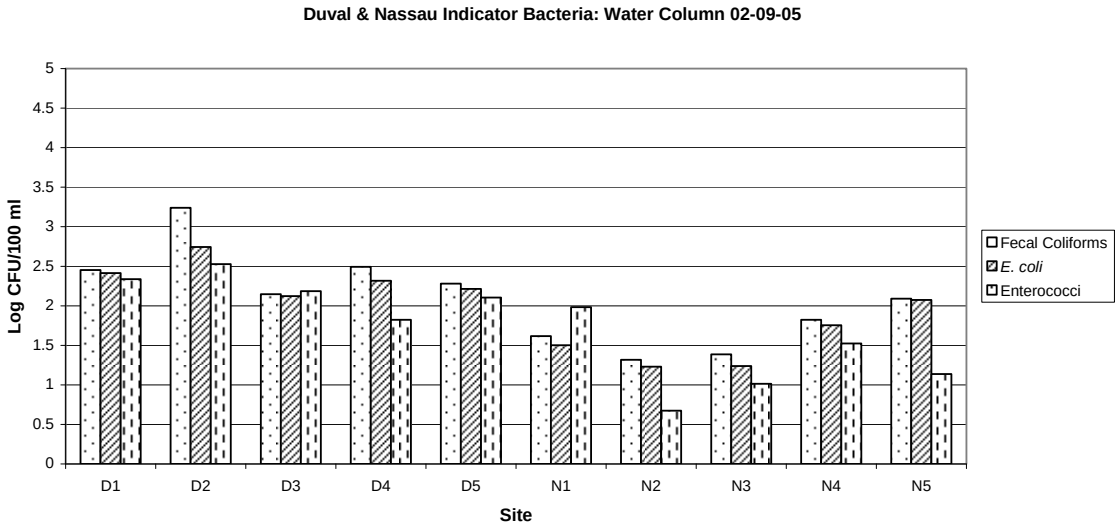


Figure A14. Indicator bacteria concentrations in sediments at the sample sites 2-09-05.

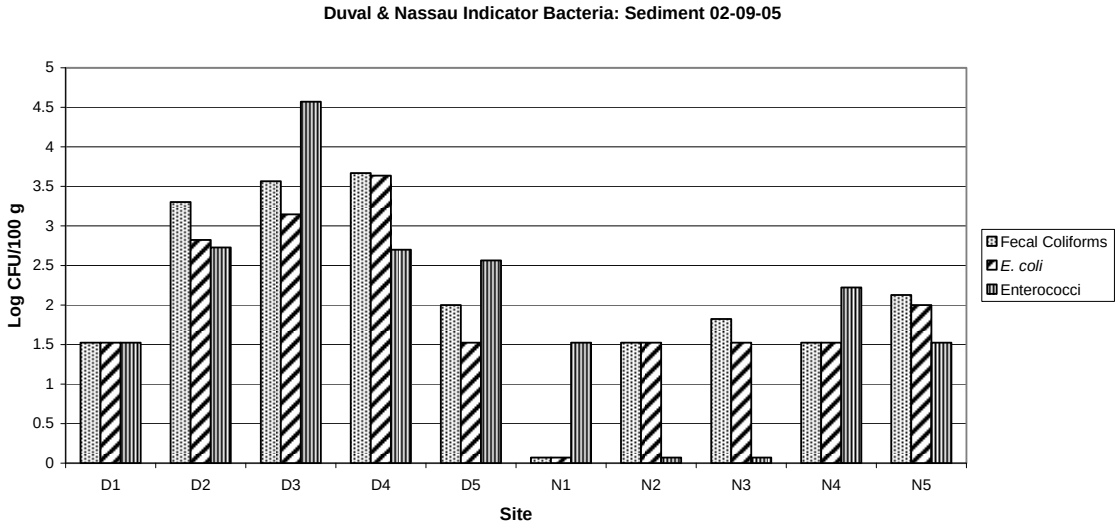


Figure A15. Indicator bacteria concentrations in the water column at the sample sites 3-14-05.

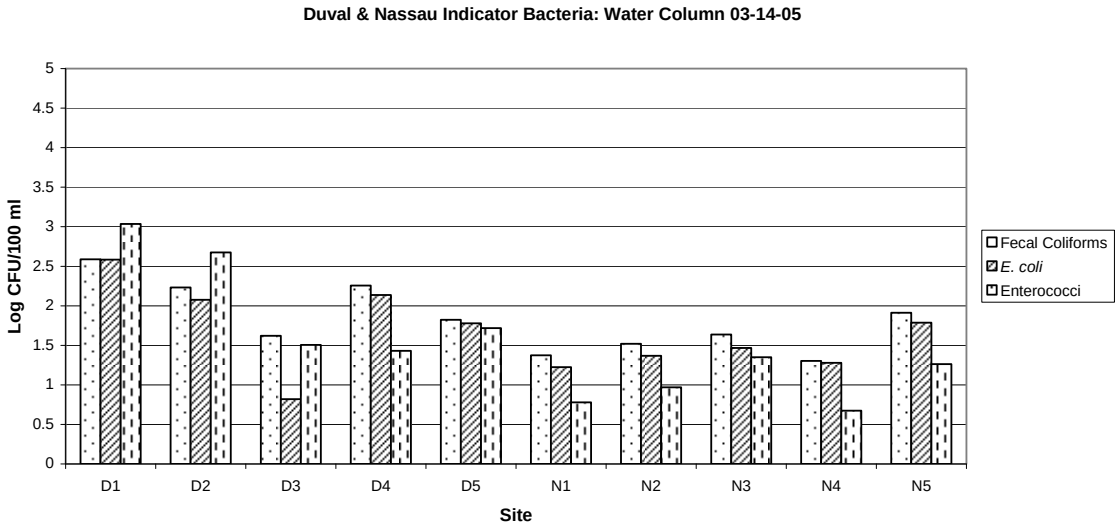


Figure A16. Indicator bacteria concentrations in sediments at the sample sites 3-14-05.

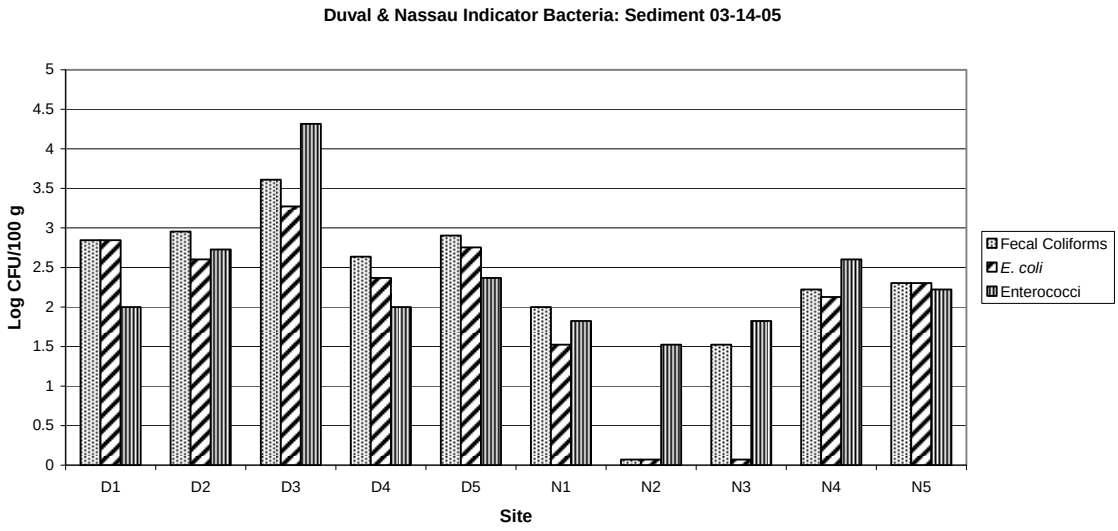


Figure A17. Indicator bacteria concentrations in the water column at the sample sites 4-18-05.

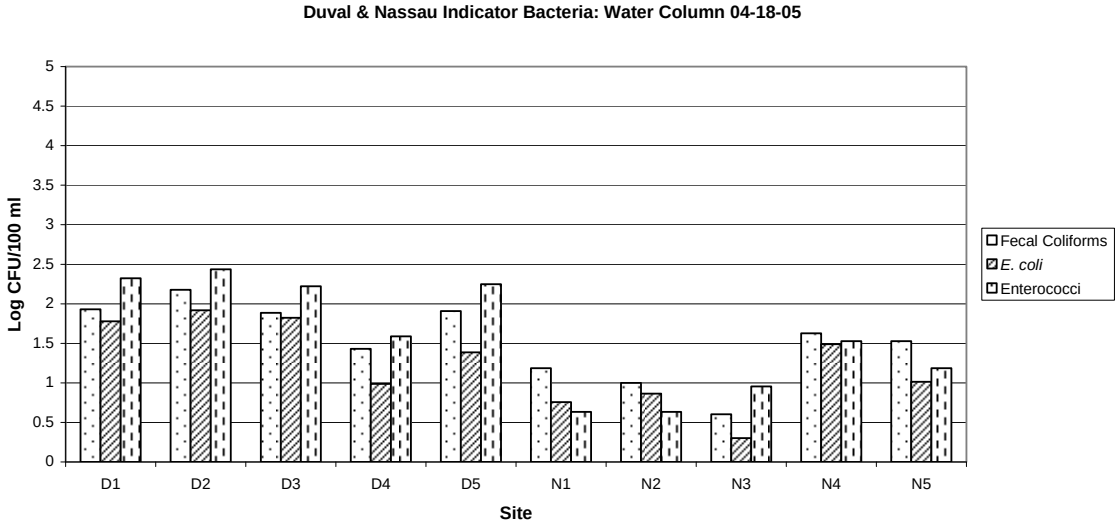


Figure A18. Indicator bacteria concentrations in sediments at the sample sites 4-18-05.

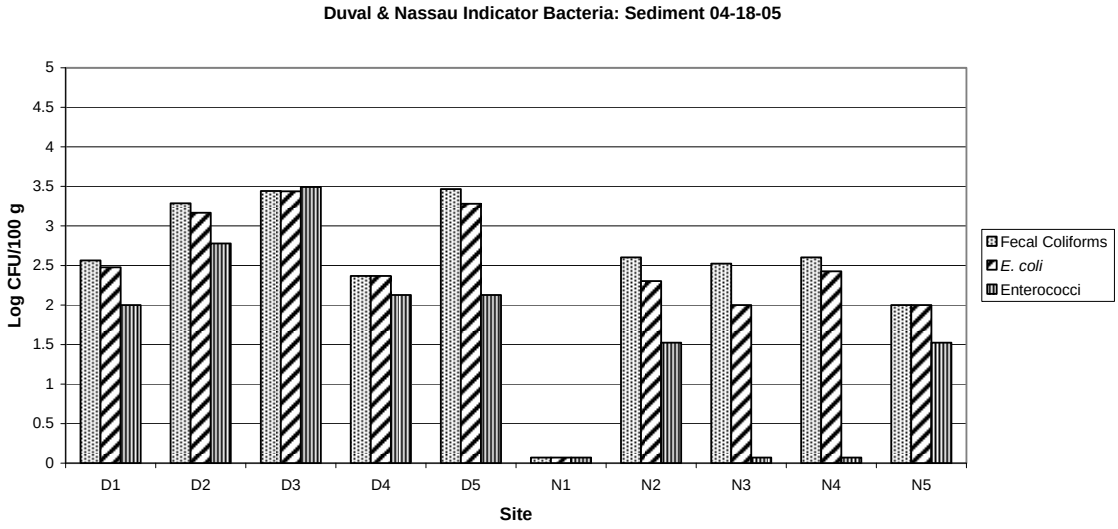


Figure A19. Indicator bacteria concentrations in the water column at the sample sites 5-09-05.

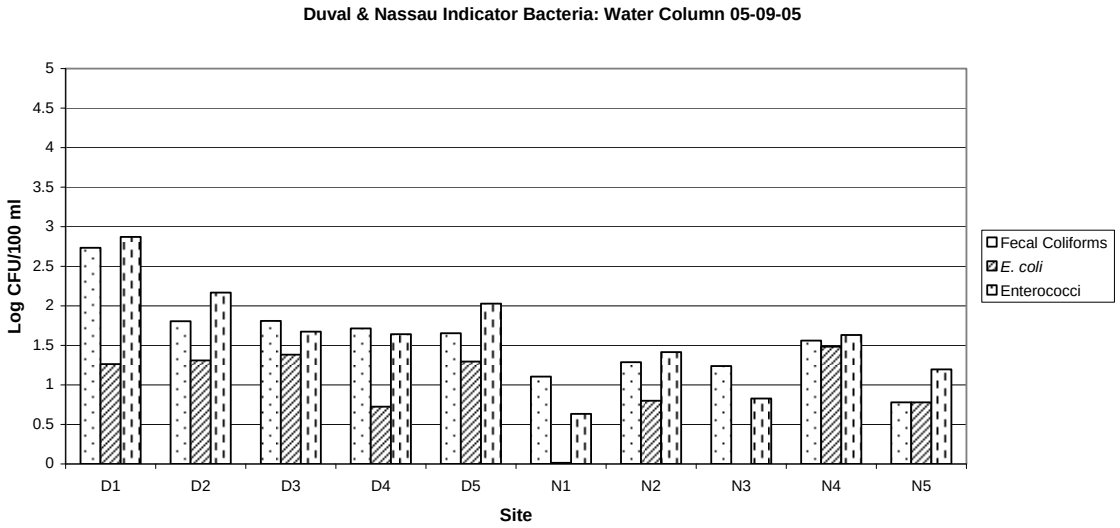
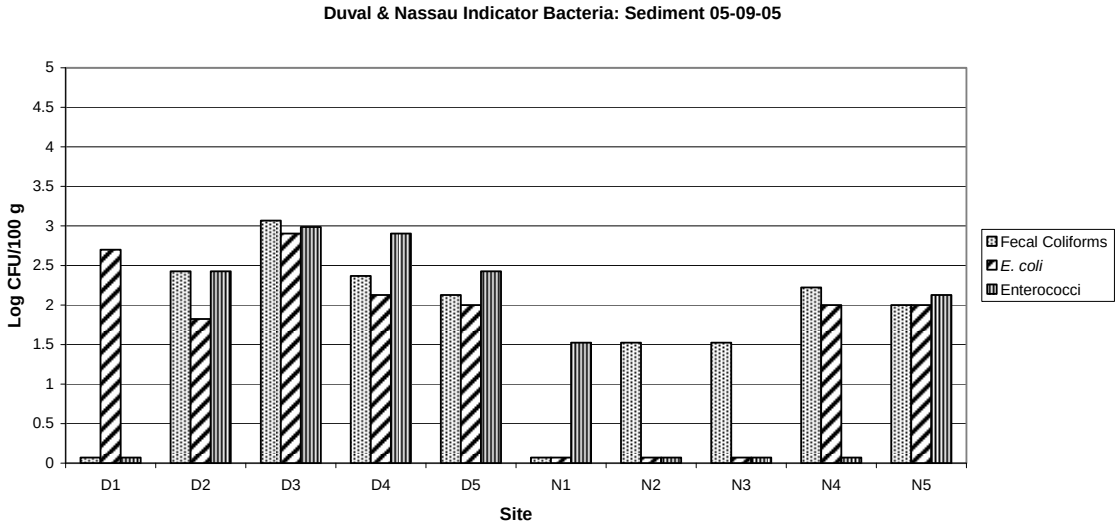


Figure A20. Indicator bacteria concentrations in sediments at the sample sites 5-09-05.



Appendix 4.

Non-library based PCR Results for USF and BCS Laboratories for each sampling event are presented on the following pages.

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 8/18/04. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
8/18/04	Duval 1	Water	Grace Lane	**(-),(+) MARKER DETECTED [1785]**	(-),(-)	(-),(-)
8/18/04	Duval 2	Water	Lenox Ave. Bridge	**(+),(+) MARKER DETECTED [2380]**	(-),(-)	(-),(-)
8/18/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-) [522.6]	(-),(-)	(-),(-)
8/18/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-) [645]	(-),(-)	(-),(-)
8/18/04	Duval 5	Water	Ortega Canal	**(+),(+) MARKER DETECTED [4500]**	(-),(-)	(-),(-)
8/18/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-) [48.6]	(-),(-)	(-),(-)
8/18/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-) [15]	(-),(-)	(-),(-)
8/18/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	**(+),(-) MARKER DETECTED [18]**	(-),(-)	(-),(-)
8/18/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(+) MARKER DETECTED [3955]**	(-),(-)	(-),(-)
8/18/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(+),(+) MARKER DETECTED [213]**	(-),(-)	(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 9/20/04. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
9/20/04	Duval 1	Water	Grace Lane	(-),(-),(-) [831.2]	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-) [1312.8]	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 3	Water	San Juan Blvd Boat Ramp	**(-),(+), (-) MARKER DETECTED [373]**	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [160]	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 5	Water	Ortega Canal	**(+),(+), (-) MARKER DETECTED [850.5]**	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-) [6.6]	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [13.4]	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [<0.6]	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [233.3]	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-) [20]	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 10/25/04. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
10/25/04	Duval 1	Water	Grace Lane	(-),(-),(-) [4100]	(-),(-),(-)	(-),(-),(-)
10/25/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-) [1149.6]	(-),(-),(-)	(-),(-),(-)
10/25/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [2716.4]	(-),(-),(-)	(-),(-),(-)
10/25/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [1860]	(-),(-),(-)	(-),(-),(-)
10/25/04	Duval 5	Water	Ortega Canal	(-),(-),(-) [877.5]	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-) [22]	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [91.2]	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [74.1]	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [166]	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-) [14]	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 11/15/04. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
11/15/04	Duval 1	Water	Grace Lane	(-),(-),(-) [4183.5]	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-) [1533.2]	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [426.6]	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [144]	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 5	Water	Ortega Canal	(-),(-),(-) [1725]	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-) [12.9]	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [15]	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [21.8]	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [122.6]	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-) [277.4]	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 12/13/04. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
12/13/04	Duval 1	Water	Grace Lane	(-),(-),(-) [2610]	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-) [2480.1]	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [1710]	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [720]	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 5	Water	Ortega Canal	(-),(-),(-) [620.1]	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-) [56.1]	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [147.9]	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [99.9]	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [2090.1]	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-) [810]	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 1/10/05. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
1/10/05	Duval 1	Water	Grace Lane	(-),(-),(-) [1530]	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-) [870]	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [939.9]	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [111.9]	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 5	Water	Ortega Canal	(-),(-),(-) [99]	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-) [27]	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [39]	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [66.9]	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [345.9]	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-) [155.1]	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 2/08/05. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
2/08/05	Duval 1	Water	Grace Lane	(-),(-),(-) [650.1]	(-),(-),(-)	(-),(-),(-)
2/08/05	Duval 2	Water	Lenox Ave. Bridge	**(+),(+),(+) MARKER DETECTED [1010.1]**	(-),(-),(-)	(-),(-),(-)
2/08/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [459.9]	(-),(-),(-)	(-),(-),(-)
2/08/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [200.1]	(-),(-),(-)	(-),(-),(-)
2/08/05	Duval 5	Water	Ortega Canal	**(-),(-),(+) MARKER DETECTED [380.1]**	(-),(-),(-)	(-),(-),(-)
2/08/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(-),(-),(+) MARKER DETECTED [288]**	(-),(-),(-)	(-),(-),(-)
2/08/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-) [14.1]	(-),(-),(-)	(-),(-),(-)
2/08/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-) [30.9]	(-),(-),(-)	(-),(-),(-)
2/08/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-) [99.9]	(-),(-),(-)	(-),(-),(-)
2/08/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(+),(+),(+) MARKER DETECTED [41.1]**	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 3/14/05. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
3/14/05	Duval 1	Water	Grace Lane	**(+),(+),(+) [3249.9]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 2	Water	Lenox Ave. Bridge	**(+),(+),(+) [1419.9]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 3	Water	San Juan Blvd Boat Ramp	**(+),(-),(-) [96]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [81]	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 5	Water	Ortega Canal	(-),(-),(-) [156]	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(+),(+),(+) [18]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	**(+),(+),(+) [27.9]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	**(+),(+),(+) [66.9]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(+),(+) [14.1]**	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(+),(+),(+) [54.9]**	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 4/18/05. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
4/18/05	Duval 1	Water	Grace Lane	**(+),(+),(+) [630]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 2	Water	Lenox Ave. Bridge	**(-),(+),(+) [819.9]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 3	Water	San Juan Blvd Boat Ramp	**(+),(-),(-) [500.1]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	**(+),(-),(-) [116.1]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 5	Water	Ortega Canal	**(-),(-),(+) [235.6]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(+),(+),(-) [12.9]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	**(-),(+),(-) [12.9]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	**(+),(+),(+) [27]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(-),(-) [73]**	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(-),(+),(+) [38.3]**	(-),(-),(-)	(-),(-),(-)

USF Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 5/09/05. Estimated CFU analyzed in ESP PCR [ ].

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
5/09/05	Duval 1	Water	Grace Lane	**(+),(+),(+) [2223]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 2	Water	Lenox Ave. Bridge	**(+),(+),(+) [440.1]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-) [141]	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-) [131.1]	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 5	Water	Ortega Canal	(-),(-),(-) [318.9]	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(+),(+),(+) [12.9]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	**(+),(+),(+) [78]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	**(+),(+),(+) [20.1]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(+),(+) [128.1]**	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(-),(-),(+) [47.1]**	(-),(-),(-)	(-),(-),(-)

USF Detection of controls (QA/QC) for host associated markers of human and ruminant fecal pollution for all sampling events.

Date Samples Collected	**Human *Enterococcus* Positive Control (*E. faecium* C68 DNA) (+/-)**	**Human *Enterococcus* Negative Control (Water) (+/-)**	**Human *Bacteroides* Positive Control (Sewage DNA) (+/-)**	**Human *Bacteroides* Negative Control (Water) (+/-)**	**Ruminant *Bacteroides* Positive Control (Cow Fecal Slurry DNA) (+/-)**	**Ruminant *Bacteroides* Negative Control (Water) (+/-)**
8/18/04	+	-	+	-	+	-
9/20/04	+	-	+	-	+	-
10/25/04	+	-	+	-	+	-
11/15/04	+	-	+	-	+	-
12/13/04	+	-	+	-	+	-
1/10/05	+	-	+	-	+	-
2/08/05	+	-	+	-	+	-
3/14/05	+	-	+	-	+	-
4/18/05	+	-	+	-	+	-
5/09/05	+	-	+	-	+	-

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 8/18/04*Single sample bottle. Three 100 ml samples analyzed from single bottle.

Date Collected	Site Number	Sample Type (Water/Sediment)	Site Location	Human *Enterococcus* Marker (+/-)	Human *Bacteroides* Marker (+/-)	Ruminant *Bacteroides* Marker (+/-)
8/18/04	Duval 1	Water*	Grace Lane	(-),(-),(-)*	(-),(-),(-)*	(-),(-),(-)*
8/18/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
8/18/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
8/18/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)	(-),(-),(-)
8/18/04	Duval 5	Water	Ortega Canal	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
8/18/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
8/18/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
8/18/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
8/18/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**
8/18/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 9/20/04

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
9/20/04	Duval 1	Water	Grace Lane	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
9/20/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 10/25/04

Date Collected	Site Number	Sample Type (Water/Sediment)	Site Location	Human *Enterococcus* Marker (+/-)	Human *Bacteroides* Marker (+/-)	Ruminant *Bacteroides* Marker (+/-)
10/25/04	Duval 1	Water	Grace Lane	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)	**(+),(+),(+) MARKER DETECTED**
10/25/04	Duval 2	Water	Lenox Ave. Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
10/25/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
10/25/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
10/25/04	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
10/25/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
10/25/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 11/15/04

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
11/15/04	Duval 1	Water	Grace Lane	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
11/15/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
11/15/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 12/13/04

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
12/13/04	Duval 1	Water	Grace Lane	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
12/13/04	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 1/10/05

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
1/10/05	Duval 1	Water	Grace Lane	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
1/10/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 2/8/05

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
2/8/05	Duval 1	Water	Grace Lane	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
2/8/05	Duval 2	Water	Lenox Ave. Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
2/8/05	Duval 3	Water	San Juan Blvd Boat Ramp	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
2/8/05	Duval 4	Water	Blanding Apt. Bldg	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
2/8/05	Duval 5	Water	Ortega Canal	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)	(-),(-),(-)
2/8/05	Nassau 1	Water	CR-125/St St. Mary's Middle Prong. Bridge	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)	(-),(-),(-)
2/8/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
2/8/05	Nassau 3	Water	CR-250/St. St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
2/8/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
2/8/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(+),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 3/14/05

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
3/14/05	Duval 1	Water*	Grace Lane	**(-),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
3/14/05	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	**(+),(+),(-) MARKER DETECTED**	(-),(-),(-)
3/14/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
3/14/05	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(-),(-),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
3/14/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	**(+),(-),(-) MARKER DETECTED**	(-),(-),(-)
3/14/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
3/14/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	**(-),(+),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 4/18/05

Date Collected	Site Number	Sample Type (Water/Sediment)	Site Location	Human *Enterococcus* Marker (+/-)	Human *Bacteroides* Marker (+/-)	Ruminant *Bacteroides* Marker (+/-)
4/18/05	Duval 1	Water*	Grace Lane	**(-),(-),(+) MARKER DETECTED**	**(+),(-),(-) MARKER DETECTED**	(-),(-),(-)
4/18/05	Duval 2	Water	Lenox Ave. Bridge	**(+),(-),(+) MARKER DETECTED**	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
4/18/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
4/18/05	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	(-),(-),(-)	**(+),(-),(-) MARKER DETECTED**	(-),(-),(-)
4/18/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	**(+),(+),(+) MARKER DETECTED**	(-),(-),(-)
4/18/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
4/18/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Detection of host associated markers of human and ruminant fecal pollution in water samples collected and assayed 5/9/05

Date Collected	**Site Number**	**Sample Type (Water/Sediment)**	**Site Location**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
5/09/05	Duval 1	Water*	Grace Lane	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 2	Water	Lenox Ave. Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 3	Water	San Juan Blvd Boat Ramp	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 4	Water	Blanding Ave. Cedar Shore Apt.Bldg.	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Duval 5	Water	Ortega Canal	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 1	Water	CR-125/St. Mary's Middle Prong Bridge	**(+),(-),(-) MARKER DETECTED**	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 2	Water	CR-127/ St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 3	Water	CR-250/St. Mary's Middle Prong Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 4	Water	Hwy 90/ Deep Creek Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)
5/09/05	Nassau 5	Water	CR-121/Brandy Branch Bridge	(-),(-),(-)	(-),(-),(-)	(-),(-),(-)

BCS Quality Control Data

Animal Source	**No. of Samples**	**Human *Enterococcus* Marker (+/-)**	**Human *Bacteroides* Marker (+/-)**	**Ruminant *Bacteroides* Marker (+/-)**
Domestic Raw Sewage	**5 (composite)**	**POSITIVE (100%)**	**POSITIVE (100%)**	NEGATIVE
Deer	**3**	NEGATIVE	NEGATIVE	**POSITIVE (100%)**
Horse	**4**	NEGATIVE	NEGATIVE	**POSITIVE (100%)**
Bird	**6**	NEGATIVE	NEGATIVE	NEGATIVE
Cattle	**7 (composite)**	NEGATIVE	NEGATIVE	**POSITIVE (100%)**
Dog	**4**	NEGATIVE	NEGATIVE	NEGATIVE

Total Number of samples in which at least one positive test was observed per site

Site	Human *Enterococcus*	Human *Bacteroides*	Ruminant *Bacteroides*	Total Human Hits (Rank 1-7)
Duval 1	5	4	1	9 (1)
Duval 2	3	4	0	7 (2)
Duval 3	1	1	0	2 (6)
Duval 4	2	1	0	3 (5 T)
Duval 5	2	1	0	3 (5 T)
Nassau 1	3	2	0	5 (3 T)
Nassau 2	1	3	0	4 (4 T)
Nassau 3	0	0	0	0 (7)
Nassau 4	2	2	1	4 (4 T)
Nassau 5	2	3	1	5 (3 T)

Appendix 5.

Validation procedures performed for non-library based MST methods.

Comparison of PCR Sensitivity for ESP and host-specific *Bacteroides* Genes between USF and BCS Laboratories

It was suggested that discrepancies between PCR assay results for BCS vs.USF analyses on the same samples (see Table 2) may be due to variations in PCR chemistry. BCS utilizes a hot start PCR method (HotStarTaq®-Qiagen). Literature from Qiagen states that the HotStarTaq and Qiagen PCR buffer minimizes non-specific amplification of products, primer dimers, and background signal. The HotStarTaq® is provided in an inactive state with no polymerase activity at ambient temperatures to prevent the formation of misprimed products and primer-dimers at low temperatures. The HotStarTaq® is activated by a 15-minute incubation at 95ºC at the beginning of the thermal cycling program. Qiagen literature does not compare sensitivity (ability to amplify small amounts of template DNA) between HotStarTaq® and conventional taq polymerase.

USF utilizes a conventional taq polymerase from Fisher Scientific for all PCR assays. The USF protocol using Fisher taq is the mainstay of the Harwood (USF) Lab and has been used successfully for many sensitive and specific PCR assays. Other than the addition of the 15-minute incubation at 95ºC at the beginning of the HotStarTaq® cycle program, the two thermocycling profiles are identical.

USF samples that showed variability between replicate samples from any site, or samples that were positive for the target marker by BCS analysis and negative from USF analysis were re-analyzed at USF using BCS's HotStarTaq® protocol for both ESP (Figure 4) and human-specific *Bacteroides* (Figure 4). Results were compared prior to and subsequently following re-analyzed samples for both ESP (Table 3) and human-specific *Bacteriodes* (Table 4) PCR.

USF-Fisher protocol:

Ingredient	For 50µl rxn
$MgCl_2$	2mM
10 X Buffer	5.0µl (1X)
dNTP mix	200µM
F- primer	0.25µM
R- primer	0.25µM
Taq	1.25U
Template DNA	5.0µl (~10-50ng)

Thermal cycle profile:

1. Initial 2 min. at 95ºC
2. 94ºC - 1 min.
3. 58ºC - 1 min. } 35 cycles (2-4)
4. 72ºC - 1 min.
5. 72ºC - 10 min. (final elongation)

BCS-HotStarTaq® protocol

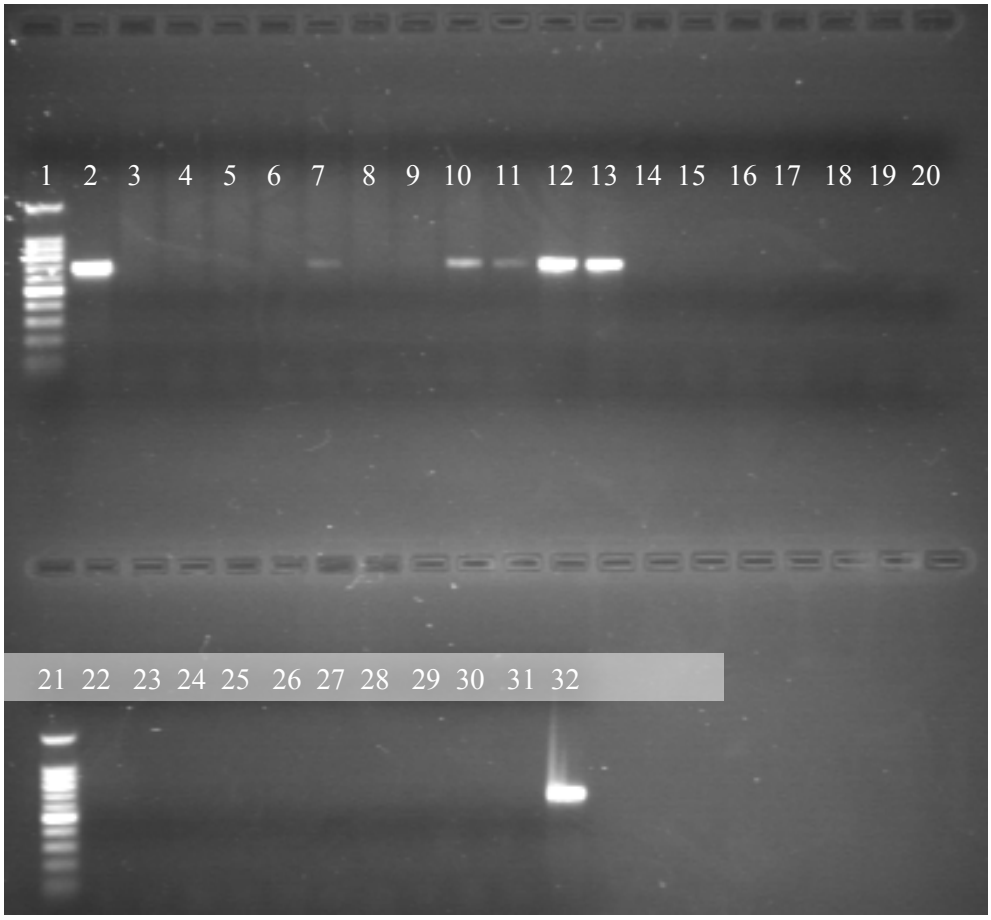
Ingredient	For 50µl rxn
$MgCl_2$	1.5mM
10 X Buffer	5.0µl (1X)
dNTP (each)	200µM
F- primer	0.3µM
R- primer	0.3µM
Taq	2.5U
Template DNA	5.0µl (~10-50ng)

Thermal cycle profile:

1. Initial 15 min. at 95ºC

2. 94ºC - 1 min.
3. 58ºC - 1 min. } 35 cycles (2-4)
4. 72ºC - 1 min.
5. 72ºC - 10 min. (final elongation)

Figure 4. Re-analyzed samples of ESP gene target using HotStarTaq®.



1. 100 bp marker
2. Positive control (*E. faecium* C68)
3. D1A 8/04
4. D1B 8/04
5. D4A 8/04
6. D4B 8/04
7. N3A 8/04
8. N3B 8/04
9. D3A 9/04
10. D3B 9/04
11. D3C 9/04
12. D5A 9/04
13. D5B 9/04
14. D5C 9/04
15. D1A 10/04
16. D1B 10/04
17. D1C 10/04
18. D2A 10/04
19. D2B 10/04
20. D2C 10/04
21. 100 bp marker
22. D4A 10/04
23. D4B 10/04
24. D4C 10/04
25. N4A 10/04
26. N4B 10/04
27. N4C 10/04
28. D1A 11/04
29. D1B 11/04
30. D1C 11/04
31. Negative control
32. Positive control (*E. faecium* C68)

Table 3. Comparison of HotStarTaq® PCR re-analyzed samples with original results using Fisher taq polymerase. Analysis was performed on USF samples that showed variability between A, B, and/or C replicates from any site, or samples that were positive for the marker by BCS analysis and negative by USF. Columns shaded yellow show discrepant results between PCR assays using the two taq preparations.

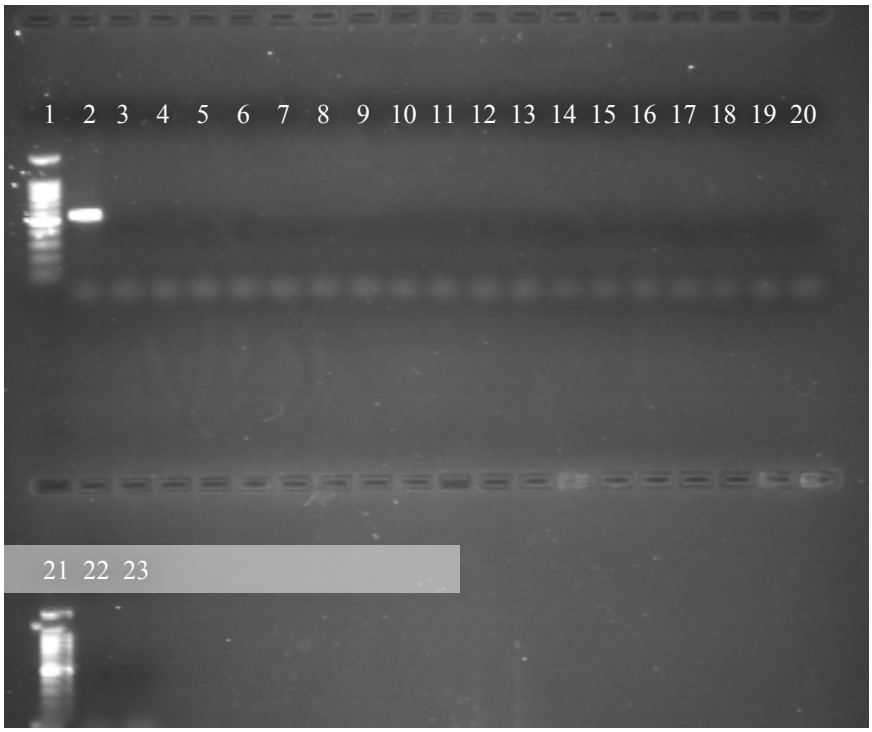
	Fisher	HotStarTaq®		Fisher	HotStarTaq®
D1A 8/04	-	-	D1C 10/04	-	-
D1B 8/04	+	-	D2A 10/04	-	-
D4A 8/04	-	-	D2B 10/04	-	-
D4B 8/04	-	-	D2C 10/04	-	-
N3A 8/04	+	+	D4A 10/04	-	-
N3B 8/04	-	-	D4B 10/04	-	-
D3A 9/04	-	-	D4C 10/04	-	-
D3B 9/04	+	+	N4A 10/04	-	-
D3C 9/04	-	+	N4B 10/04	-	-
D5A 9/04	+	+	N4C 10/04	-	-
D5B 9/04	+	+	D1A 11/04	-	-
D5C 9/04	-	-	D1B 11/04	-	-
D1A 10/04	-	-	D1C 11/04	-	-
D1B 10/04	-	-			

RED- USF samples that showed variability between A, B, and/or C of any site.
BLUE- Samples that were positive for the marker at BCS and negative at USF.

None of the BCS-positive samples that were negative at USF (blue) were positive in the HotStar or Fisher PCR assays. This result does not support the hypothesis that use of HotStarTaq® by BCS led to the discrepant results. Replicate samples that were inconsistent when first analyzed (red) were not more often amplified by HotStarTaq® than by Fisher taq. Again, this result does not support the hypothesis that use of HotStarTaq® is causing the discrepancy between USF's inconsistent PCR results and BCS's consistent PCR results.

Similar quality control analysis was run with the *Bacteroides* human-specific primers.

Figure 5. Re-analyzed samples for human-specific *Bacteroides* PCR using HotStarTaq®.



1. 100 bp marker
2. Positive control (*E. faecium* C68)
3. D5A 8/04
4. D5B 8/04
5. N4A 8/04
6. N4B 8/04
7. D2A 10/04
8. D2B 10/04
9. D2C 10/04
10. D4A 10/04
11. D4B 10/04
12. D4C 10/04
13. N4A 10/04
14. N4B 10/04
15. N4C 10/04
16. N5A 10/04
17. N5B 10/04
18. N5C 10/04
19. D1A 11/04
20. D1B 11/04
21. 100 bp marker
22. D1C 11/04
23. Negative control

Table 4. HotStarTaq® PCR analysis on USF discrepant samples. All samples were positive at BCS and negative at USF using both Fisher and HotStarTaq®.

	Fisher	HotStarTaq®		Fisher	HotStarTaq®
D5A 8/04	-	-	N4A 10/04	-	-
D5B 8/04	-	-	N4B 10/04	-	-
N4A 8/04	-	-	N4C 10/04	-	-
N4B 8/04	-	-	N5A 10/04	-	-
D2A 10/04	-	-	N5B 10/04	-	-
D2B 10/04	-	-	N5C 10/04	-	-
D2C 10/04	-	-	D1A 11/04	-	-
D4A 10/04	-	-	D1B 11/04	-	-
D4B 10/04	-	-	D1C 11/04	-	-
D4C 10/04	-	-			

*There is a noted sample volume discrepancy between BCS and USF (USF processed ~ 1/3 the volume of water than BCS) on all dates prior to 02/05.

As noted for the ESP PCR assays, PCR using HotStar Taq on discrepant *Bacteroides* samples did not yield any additional positive PCR results than Fisher taq (all assays were negative).

Amplification sensitivity of dilute target concentrations: BCS HotStarTaq® vs USF ESP protocols. The sensitivity of the USF PCR protocol and the BCS HotStarTaq® protocols were compared by running two dilution series of *E. faecium* C68 (1) control template. 5µl of a 1:2 and 1:10 dilution series (Figures 6 and 7) was used as template for both protocols. Theoretical cell numbers were calculated for each volume of purified genomic DNA template used for each dilution. The HotStarTaq® BCS protocol was able to detect DNA from as few as ~4.42×10^2 *E. faecium* cells, whereas the Fisher USF protocol was able to detect DNA from as few as ~4.42×10^1 cells (Figure 7).

Figure 6. 1:2 dilution series: HotStarTaq® vs. Fisher Taq

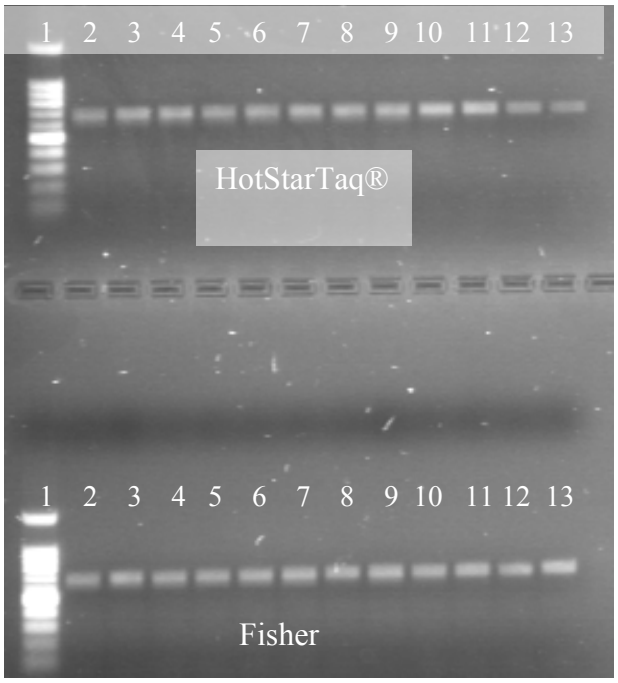
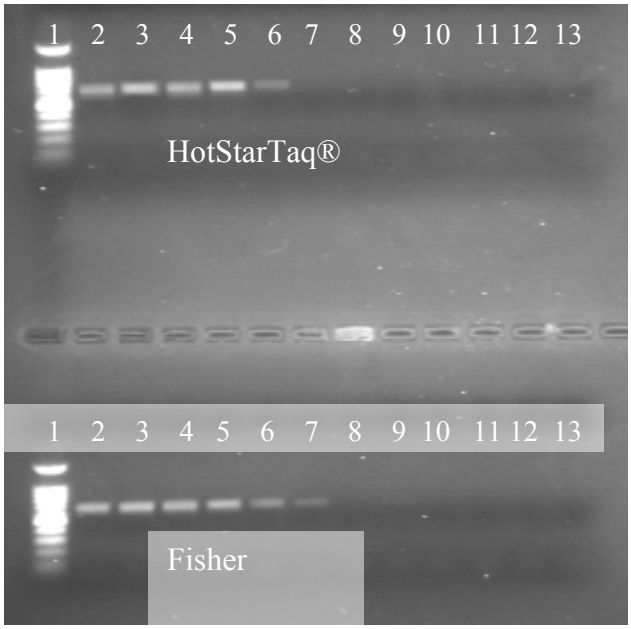
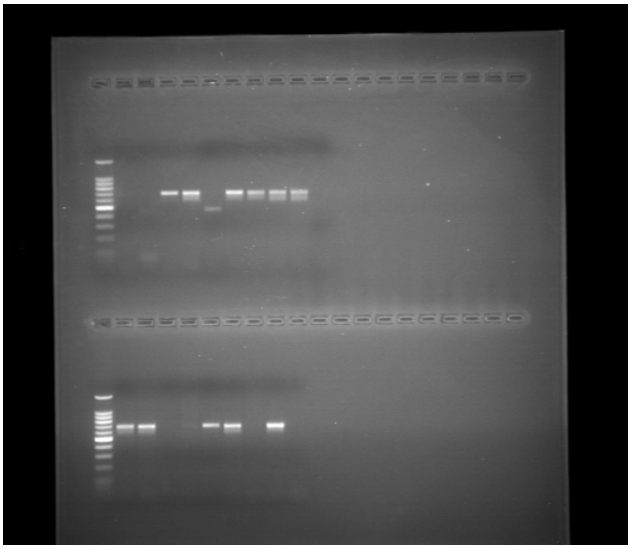


Figure 7. 1:10 dilution series: HotStarTaq® vs. Fisher Taq



These results do not support the hypothesis that PCR discrepancies (specifically USF nondetects for samples in which BCS records detects) are due to the sensitivity of HotStarTaq®. We are in the process of setting up a laboratory exchange, in which BCS personnel will visit the USF laboratory with discrepant samples and controls, and vice versa to further investigate the cause(s) of the discrepant samples.

Blind Test Samples (ESP PCR- 03/14/05)



Top Lanes: 1- 100 bp ladder
2- Tube 1
3- Tube 2
4- Tube 3
5- Tube 4
6- Tube 5
7- Tube 6
8- C68 DNA (+)
9- C68 DNA (+)
10- C68 DNA (+)
11- Negative Control
12-15 Empty

Bottom Lanes: 1- 100 bp ladder
2- C68 DNA (+)
3- C68 DNA (+)
4- Tube 1
5- Tube 2
6- Tube 3
7- Tube 4
8- Tube 5
9- Tube 6
10- Negative Control
9- C68 DNA (+)
10- C68 DNA (+)
11-15 Empty

****Note: Cultures in tubes 1, 3, 4, and 6 were very dark brown (and smelled awful), while those in 2 and 5 were milky in color.

Samples arrived from BCS via FedEx at 11:10am. DNA extraction using Qiagen Stool Kit performed immediately.

Quantified DNA PCR & Hot Star PCR initiated at 6:00pm on 3/14/05. Gel was started at 11:00am (1.5% in TAE; run at 95V for 1h)

Fisher Taq recipe using 50ng of template (**quantified** DNA) for one 50µl reaction:

Ingredient	For 50µl rxn
$MgCl_2$	2mM
10 X Buffer	5.0µl (1X)
dNTP mix	200µM
F- primer	0.25µM
R- primer	0.25µM
Taq	1.25U
Template DNA	5.0µl (~10-50ng)

Hot Star PCR recipe using 5µl of template for one 50µl reaction:

Ingredient	For 50µl rxn
$MgCl_2$	1.5mM
10 X Buffer	5.0µl (1X)
dNTP (each)	200µM
F- primer	0.3µM
R- primer	0.3µM
Taq	2.5U
Template DNA	5.0µl

* All reagents are stock straight from the freezer.

Hot Star PCR Program:

6. Initial - 2 min. at 95ºC
7. 94ºC - 1 min.
8. 58ºC - 1 min. } 35 cycles
9. 72ºC - 1 min.
10. 72ºC - 10 min. (final elongation)

PCR Program:

1. Initial - 2 min. at 95ºC
2. 94ºC - 1 min.
3. 58ºC - 1 min. } 35 cycles
4. 72ºC - 1 min.
5. 72ºC - 10 min. (final elongation)

Appendix 6.

Logistical and methodological challenges to implementing new MST approaches, and associated strategies for overcoming these challenges.

Method	Specific Challenge	Strategy for Correction	Result
BOX-PCR typing	Inconsistent DNA yield from cultures	Use of Promega Wizard SV 96 Genomic DNA Purification System)	Consistent DNA yield for both laboratories
BOX-PCR typing	Poor resolution of patterns on gel (BCS)	Technician trained in USF lab	Good pattern resolution with results that could be shared between laboratories
BOX-PCR Library Analysis	Poor agreement of Jackknife analysis between labs (BCS)	Technician trained in USF lab on proper statistical analysis for library development/comparisons	Good library identification with results that could be shared between laboratories
esp PCR (human)	False-positive results caused by DNA contamination (USF)	Rearrangement of physical set-up of laboratory	Negative controls clean, and artificially elevated percentage of positive samples reduced.
Bacteroides PCR (human or ruminant)	No positive results from samples, although positive controls working after alteration to amended method (USF)	Modification of DNA isolation protocol	Good agreement for positive samples between laboratories after USF use of amended method in a subsequent/on-going project in Jacksonville.

IX. Laboratory Quality Assurance/Quality Control Plan

Water Quality Monitoring/Microbial Ecology Laboratory
Valerie J. Harwood, Ph.D.- Principle Investigator

A. Quality Policy Statement and Commitments by Top Management

The laboratory is committed to upholding the highest degree of professionalism and expertise in all aspects of Environmental and Molecular Microbiology. The laboratory focuses on identification of microbial indicators found in water and wastewater, as well as in identification of potential sources of fecal contamination (Microbial Source Tracking) and microbial population dynamics in environmental waters.

B. Identification of approved signatories for the laboratory

Dr. Harwood prepares, oversees and validates final results, supervises analyses, and directs the environmental and molecular laboratories.

All laboratory reports will be signed and approved by Dr. Valerie J. Harwood

C. List of all Test Methods under which testing is being performed

Standard Operating Procedures – All standard operating procedures (EPA methods, Standard Methods) are available to all personnel in the SOP notebook or in reference manuals.

1. **SM9222D (Fecal Coliform Bacteria) Membrane Filtration Method in accordance with Standard Methods for the Examination of Water and Wastewater.**

   Summary of Method:
   Fecal coliforms are analyzed by the membrane filtration technique using membrane fecal coliform (mFC) media. The medium is prepared by dissolving 52 g of dehydrated medium per liter of deionized water, followed by heating while stirring with a magnetic stir bar. The suspension is boiled for one minute, followed by the addition of 10 ml 1% rosalic acid in 0.2 N NaOH per liter. Liquefied media is dispensed into plates, which are kept refrigerated for up to 2 weeks.

   For analysis of water samples or sonicated sediment samples that have been passed through membrane filters, each filter is placed on an mFC media plate. The plates are placed into whirl-pack bags with waterproof enclosures and incubated submerged in a water bath at 44.5 $\pm$ 0.2° C for 24 +/- 2 hours. Blue colonies are counted as fecal coliforms. Pink, cream, gray or other non-blue colored colonies are not considered fecal coliforms.

   Quality Control:
   Escherichia coli C-3000 (ATCC 15597) is used as a positive control for verification of media and processing integrity. Colonies that grow and exhibit dark blue pigmentation are considered as positive verification of fecal coliform bacteria. Filtering sterile

buffered water through a membrane filter and incubating the media along with positive control sample serves as a negative control.

2. SM9230C (*Enterococcus spp.*) or EPA Method 1600 - Membrane Filtration Method in accordance with the Clean Water Act and Standard Methods for the Examination of Water and Wastewater.

Summary of Method:
The medium used in this assay is mEI agar, which is prepared by dissolving 71.2 g dehydrated mE agar (Difco) and 750 mg indoxyl β-D-glucoside per liter deionized water and autoclaving for 15 minutes at 121° C. Autoclaved media is cooled to 45-50° C in a water bath, and to each liter of media is added 10 ml of a 24 mg/ml nalidixic acid solution and 0.2 ml of a 10% 2,3,5-triphenyltetrazolium chloride (TTC) solution; both reagent supplements are dissolved in sterile deionized water. Media is dispensed into plates and allowed to solidify. Plates are stored in the dark at 4^{o}C and kept for a maximum of two weeks.

After water samples or sonicated sediment samples have been passed through membrane filters, filters are placed on mEI media plates and incubated at 41° C for 24 +/- 2 hours. Enterococci colonies are small, gray colonies with a blue fringe. Only colonies with this appearance are counted as enterococci.

Quality Control:
Enterococcus faecalis (ATCC 35550) is used as a positive control for verification of media and processing integrity. Colonies that grow and exhibit dark blue to blue-gray pigmentation are considered as positive verification of *Enterococcus spp*. Filtering sterile buffered water through a membrane filter and incubating the media along with positive control sample serves as a negative control.

3. Modified EPA Method 1103 - Membrane Filtration Method for *Escherichia coli*.

Summary of Method:
E. coli are analyzed by membrane filtration using mTEC agar plates. The medium is prepared by mixing 45.6 g of dehydrated mTEC agar per liter of deionized water. The suspension is dissolved by boiling while stirring with a magnetic stir bar, and sterilized by autoclaving for 15 minutes at 121° C. Media is then dispensed into sterile petri dishes and solidified agar plates are stored in the refrigerator for a maximum of two weeks.

After water samples or sonicated sediment samples have been passed through membrane filters, each filter is placed on an mTEC media plate and incubated for 2 hours at 35 $\pm$ 0.2° C, followed by 22-24 hours at 44.5 $\pm$ 0.2° C, submerged in a water bath. After incubation, red or magenta colonies are counted as *E. coli*.

Quality Control:

Escherichia coli C-3000 (ATCC 15597) is used as a positive control for verification of media and processing integrity. Colonies that grow and are red/magenta are considered as a positive result for *E. coli* using mTEC medium. Filtering sterile buffered water through a membrane filter and incubating the media along with positive control sample serves as a negative control.

4. Overall Quality Control for Membrane Filtration Analyses

Membrane Filters – Upon receipt, each lot number of membrane filters is logged and tested for sterility by placing filter on Tryptic Soy Agar (TSA) and incubating at 35^{o}C for 24 hours.

At least once per year, each analyst must successfully perform a blind sample and/or authentic sample that is known or has been performed by another trained analyst with statistically similar results.

D. Laboratory Equipment and Calibration and/or Verification of Test Procedures Used

1. Laboratory equipment

The facility is equipped with a full-scale laboratory capable of performing a wide variety of analyses. The laboratory has a total of ~1200 sq. ft of research space. Equipment includes: an autoclave, high speed refrigerated centrifuges, microcentrifuges, a deionized water system, Reagent grade (Milli-Q) water system, refrigerated recirculating water bath, fecal coliform recirculating water bath, electrophoretic power and associated gel supplies, PCR thermocyclers, fluorometers, incubators, balances, pH meters, -80^{o}C freezers, refrigerators/freezers, mixing platforms, and UV transilluminators. All are routinely certified, monitored, and/or calibrated.

2. Calibration and Maintenance of Laboratory equipment

2.1 pH meters - All pH meters are calibrated within $\pm$ 0.1 units using two point calibration (4.0, 7.0, 10.0) prior to each use. All pH calibration buffers (NIST Traceable) are aliquotted and used only once and stocks are discarded upon expiration. Electrodes are maintained according to manufacturer's instructions.

2.2 Balances - All balances are calibrated monthly using ASTM (NIST traceable) type weights. In addition, professional calibration of all balances occurs at least once annually.

2.3 Incubators - All incubators are maintained at their desired temperature $\pm$ 0.5 ^{o}C or $\pm$0.2 ^{o}C, depending on application. Incubator temperatures are monitored using bulb thermometers immersed in glycerol, which are calibrated by a NIST traceable thermometer. Temperatures are recorded daily on log sheets.

2.4 Autoclave - Each autoclave cycle is recorded in a log book that indicates the date, contents, sterilization time, temperature, and analyst's initials. Sterilization efficiency is monitored monthly using spore ampules of *Bacillus stearothermophilus* as a control.

2.5 Sterilization procedures - All items are sterilized in the autoclave at 121^{o}C for a minimum of 15 minutes. Biohazardous wastes are sterilized for a minimum of 30 minutes.

2.6 Refrigerators - All refrigerators/freezers are monitored to maintain a temperature of 1-8 ^{o}C or -20 to -15^{o}C, respectively, by a bulb thermometer immersed in glycerol.

3. Procedures for Achieving Traceability of Measurements

All measurements by analytical equipment are recorded and dated by each user after use. Log sheets are filed for reference for up to 3 years.

4. Quality assurance of accuracy and precision of data

Quality assurance (Internal standards, duplicate samples) measures are listed with individual SOPs within the QA document.

E. Laboratory setup and procedure

1. Laboratory setup and environment

1.1 Bench space - All laboratory areas have sufficient bench space for reagent and supply storage and operation of equipment. Excess space is available for performing laboratory work.

1.2 Lighting - sufficient overhead fluorescent lighting is present in each room. Emergency lighting that has its own power supply is also present in each room.

1.3 Waste disposal - Routine materials are placed in trashcans; infectious wastes and potential pathogens are collected in specialized containers and marked to be sent for incineration.

1.4 Safety considerations - General safety procedures are followed: Lab coats and gloves are worn. Chemical waste is stored in designated containers and appropriate safety cabinets are used for storage of chemicals.

1.5 Chemicals - All chemicals and reagents are stored in clearly labeled bottles and labeled with date received and opened and are discarded according to manufacturer's instructions. Precautions and reactivity are indicated on storage containers. Chemical waste is stored in designated labeled containers and sent for appropriate disposal. Safety cabinets are used for storage of chemicals. Materials Safety and Data Sheets (MSDS) are filed and are available for reference by lab personnel.

2. Bacteriological assays

2.1 Grab Sampling – Water samples for bacteriological assays are collected by the grab sample method as in Standard Methods for the Examination of Water and Wastewater (9060A). Polyethylene bottles are pre-sterilized by autoclaving and closed with a screw-cap lid. Sampling technicians are to wear latex gloves and change gloves between each sample collection. All specimens collected are labeled properly in the field with sampling site, date and time of collection and initials of technician collecting. Samples will be kept on ice until delivery at the laboratory for processing. A field log sheet shall accompany all samples with all needed information documented on the form the sample. The time specimens are received in the laboratory is also documented on the field log sheet along with the initials of person receiving specimens.

Sediment Sampling- A Ponar is used to collect sediment samples and pre-sterilized polyethylene 50ml bottles are used to collect 40 g of the sediment sample from the Ponar. Sampling technicians are to wear latex gloves and change gloves between each sample collection. All specimens collected are labeled properly in the field with sampling site, date and time of collection and initials of technician collecting. Samples will be kept on ice until delivery at the laboratory for processing. A field log sheet shall accompany all samples with all needed information documented on the form the sample. The time specimens are received in the laboratory is also documented on the field log sheet along with the initials of person receiving specimens. Sediment samples are processed by adding 10 g of sediment per 100 mL of buffered water (0.0425 g L-1 KH_2PO_4 and 0.4055 g L-1 $MgCl_2$) followed by sonication of the mixture using an ultrasonic dismembrator (Fisher Scientific, model 100) for 30 seconds at 14 watts. The samples are allowed to settle for 10 minutes, and the supernatant is removed for filtration.

2.2 Membrane Filtration equipment - All membrane filtration manifolds are constructed from reinforced plastic and are verified for proficiency by authorized laboratory personnel prior to use. Pumps are also inspected and cleaned on a bimonthly basis to ensure proper functioning of the equipment.

2.3 Membrane filters - All filters are composed of cellulose ester fibers. They are white, grid-marked, 47mm in diameter, 0.45μm pore size, and purchased pre-sterilized.

2.4 Petri dishes - Presterilized plastic petri dishes (filled with the appropriate medium) are used for routine bacterial analyses using membrane filtration.

2.5 Sample containers - Sample containers are wide mouth plastic bottles with airtight caps or presterilized polyethylene 50 ml test tubes with airtight caps.

2.6 Laboratory bacterial control strains - Positive controls for the various assays are the following: Fecal coliform bacteria and *E. coli* – *E. coli* ATCC #15597, Enterococci – *Enterococcus faecalis* ATCC #19433

2.5 Sample containers - Sample containers are wide mouth plastic bottles with airtight caps or presterilized polyethylene 50 ml test tubes with airtight caps.

2.6 Laboratory bacterial control strains - Positive controls for the various assays are the following: Fecal coliform bacteria and *E. coli – E. coli* ATCC #15597, Enterococci – *Enterococcus faecalis* ATCC #19433

Stocks are obtained from the American Type Culture Collection and maintained by initially re-hydrating the freeze-dried culture and propagating according to ATCC instructions for each organism. Once a high-concentration broth culture of the organism has been grown, 500 µl aliquots of the suspension are mixed with 6 drops of glycerol in 1 ml cryovials and preserved at -80° C.

3. Molecular Biology Quality Control

3.1 Analyses- Molecular biology (PCR, Rep-PCR) is performed in an isolation room separated from live bacterial cultures and free DNA.

Experiments are performed in a UV cabinet and all equipment is exposed to ultraviolet radiation for a minimum of 15 minutes before use. Analyses are performed using separate autoclavable pipettors with aerosol resistant tips and latex gloves.

3.2 Laboratory bacterial control strains - Negative and positive controls are used in all PCR reactions. Negative controls consist of reactions containing no template DNA and only water. Positive controls for the various assays are as follows: ESP- *Enterococcus faecium* C68 and BOX-PCR- *E. faecalis ATCC 19433*.

F. DNA Extraction

DNA is extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions for Gram-negative and Gram-positive bacteria.

1. Determination of DNA Concentration

DNA concentration is determined using a Beckman DU 640 spectrophotometer according to manufacturer's instructions

G. Data reporting and Statistical Analysis

All data will be entered in Data Log Sheets (DLS) and transferred to computer spreadsheet files for analysis. DLSs will be kept in a binder in the laboratory. At their weekly meeting, Dr. Harwood and Dr. Shehane will confirm that the data has been

correctly transferred from DLS sheets to computer spreadsheets.

ANOVA will be used to compare variation in indicator organism concentrations between sites. The SPSS program will be used for ANOVA and related analyses, including linear regression. Linear regression will be used to correlate indicator organism concentrations to watershed impact level. Binary logistic regression will be used to determine correlations between indicator values and binary data such as the presence/absence of human markers. Discriminant analysis will be used for multivariate analysis of many indicator parameters against a specific outcome (e.g. presence of human-specific markers).

I. Procedure for Handling Collected Samples

1. Transport of Samples

All samples are received cold or on ice and temperature is verified upon receipt by measuring temperature of ice or water in the shipping container. Once received, samples are immediately labeled, recorded, processed, and then stored in the refrigerator until the following day to ensure proper sample analysis.

2. Holding times

All bacteriological samples are stored for a maximum of 12 hours.

3. Sample storage

Water samples are maintained at $4^{o}C$ and analyzed upon receipt.

4. Record keeping

Laboratory worksheets and notebooks are maintained to record sample information. Sample information is recorded and contains the following information:

4.1. Name of sampling site
4.2. Sample identification code
4.3. Sample type (water, sediment, etc.)
4.4. Date and time of collection
4.5. Analyses to be performed
4.7. Name of technician and organization
4.8. Transportation condition (temperature, etc.)

5. Chain of Custody Forms

Chain of custody forms are used when samples are transferred between parties. These forms follow state-applicable guidelines and are filed upon receipt.

J. Corrective action contingencies

1. Unacceptable results

If unacceptable results are obtained, tests with additional positive and negative controls are conducted after calibration of all equipment used in the procedure to determine the source of the problem

2. Departure from documented procedures or standard specifications

If a methodology is deemed inaccurate or unreliable for a particular sample, alternative methodologies will be independently pursued. If results from new procedures are

consistent, standard operating procedures may be modified. Dr. Harwood must approve deviations from standard procedures.

K. Procedures for data reduction, verification, validation, and reporting of results

1. Data reduction

All statistical analyses are performed using analytical computer software. Results are compiled into reports and are stored as a hard copy and in a computer database, and backed up by external electronic storage devices.

2. Accuracy of transcriptions

Sample collection sheets and laboratory data sheets are compared and verified before report preparation and are saved and available for confirmation of results.

3. Data Validation

Dr. Harwood will monitor compliance with internal audits.

4. Reporting

Copies of all data, reports, and monitoring forms, as well as final reports, are supplied to the primary investigator, Dr. Harwood, and filed for further use.

L. Procedures for training new personnel

1. Training of personnel

Dr. Harwood and/or the senior postdoctoral associate or technician trains all personnel on the proper use of all equipment prior to beginning work.

2. Training on new equipment or procedures

All personnel are trained on new equipment or procedures, as necessary. All personnel are tested on their knowledge base, and are trained and familiarized with standard research and safety practices.

3. Training on ethical and legal responsibilities

All personnel are trained on proper laboratory procedures with regards to ethical and legal rights and responsibilities, according to University of South Florida guidelines.

4. Access to QA/QC procedures

All lab personnel are provided with access to the Laboratory QA/QC plan. All personnel are required to read the document before beginning work. Revisions to the plan are

documented with date and are recorded directly on the document.

M. Record keeping and reporting of results

1. Record keeping

Records are maintained in bound notebooks and on the College of Arts and Sciences server, as well as on CDs. All records are stored for a minimum of 5 years. Records include raw data, calculations, and quality control data.

2. Reporting of Results

Results are reported as direct quantitative counts or as probable pollution source. Reports include methodology used, positive and negative controls used, overall results, and interpretation of final results.

N. Appendix

Sample Collection and Processing Forms:

1. Physical/Chemical Water Quality Field Data Sheet
2. Microbial Indicator Processing and Raw Data Sheet

Field Data Sheet: Water Quality Laboratory/Microbial Ecology Lab., USF Department of Biology
Project Name: _____

Sampling Date: _____ Personnel_____

Sampling site	Sample Type	Time	Temp	Salinity	pH	Turbidity	DO	UV	Comments

Time and Date received in the laboratory_____

Received by _____

Raw Data Sheet for Bacterial Indicators Water Quality Laboratory – USF Department of Biology

Project Name _____

Organism _____ Date/Time of Sampling _____

Date/Time processed _____ Tech _____ Incubator Temp _____

Date/Time placed in incubator _____ Date/Time removed from incubator_____

Media used _____ Date of media production/Tech _____

	Volume filtered or Dilution made (Run in duplicate) Dilutions:														**Calculations (CFU/100mL)**	
Site	100	100	50	50	25	25	10	10	5	5	1	1			**Average**	**Final Count**

Positive Control:
Negative Control:

VII. Acknowledgements.

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