APPLICATION OF MICROBIAL SOURCE TRACKING (MST) METHODS IN ASSESSMENT OF THE SOURCES OF FECAL POLLUTION IN TRIBUTARIES

Cheryl M. Wapnick[2], Asja Korajkic[1] and Valerie J. Harwood[1*]

[1]Department of Biology, University of South Florida, Tampa, FL
[2]PBS&J, Jacksonville, FL

[*]Corresponding author
Department of Biology, SCA 110
University of South Florida
4202 E. Fowler Ave.
Tampa, FL 33620

ABSTRACT

Microbial source tracking (MST) methods designed to discriminate between human versus animal fecal material were applied to the assessment of surface water quality of seven tributaries of St. Johns River in Duval County, FL. The goal was to collaborate with local agencies and stakeholders to develop and evaluate a methodology for assessing the source(s) of fecal pollution. In this study, indicator organisms (IOs) were quantified by standard membrane filtration methods in river waters and sediments. Florida state standards for IO concentrations in recreational waters were frequently exceeded (57.7% and 83.9% of samples) for fecal coliforms and enterococci, respectively. Although no regulatory standards exist for IO concentrations in the sediments, the majority of samples contained high levels of all the indicators. Four library-independent MST methods were employed at specific sites based on an adaptive, decision tree-based approach, in which results from previous sample events were used to determine subsequent sampling strategies. The MST methods relied on polymerase chain reaction (PCR) assays targeting human-associated (*esp* gene of *Enterococcus faecium*, and 16S rRNA genes of *Ent. faecalis* and *Bacteroides* spp.) and ruminant-associated (*Bacteroides* spp.) genetic markers. The frequency of detection of human-associated markers across all samples was 37.4% (*E. faecalis*), 57.1% (*Bacteroides* spp.), and 60.0% (*esp*), while the ruminant marker was detected in 45.5% of samples. In cases where library-independent methods failed to identify a pollution source, a library-dependent method utilizing BOX-PCR fingerprints of enterococci isolates was used in an attempt to determine whether sediment reservoirs of enterococci, rather than a fresh source of fecal contamination, were impacting water quality. The combination of using local GIS information and knowledge of the tributaries, together with library-dependent and library-independent MST methods, was able to identify pollution sources impacting the tributaries in most cases. This approach demonstrates the advantages of collaboration between academic groups and consultants in addressing microbial water quality issues in surface waters. The culmination of this effort is the *Tributary Pollution Assessment Manual* that provides a blueprint for conducting site assessments to locate dominant sources of fecal contamination, identify appropriate corrective actions to sufficiently reduce fecal coliform levels, and restore the WBIDs to their designated recreational use. Since most of these WBIDs will have a total maximum daily load (TMDL) for fecal coliforms established by the State, both the specific MST results of this project and the *Tributary Pollution Assessment Manual* have already begun to serve as integral parts of the Basin Management Action Plan for these tributaries, and the Manual is being used as a blueprint for similar studies conducted in other areas of Florida.

INTRODUCTION

Objective. The St. Johns River in Duval County is influenced by a very large watershed that is characterized by land use ranging from urban, to agriculture and undeveloped lands. Fifty-one of the 194 tributary water body IDs (WBIDS) to the St. Johns River in Duval County do not meet State of Florida water quality standards for fecal coliform contamination and are listed as impaired waters. PBS&J was contracted to work in cooperation with the Tributary Assessment Team (TAT) in Jacksonville toward the goal of sufficiently reducing fecal coliform levels in impaired water body segments to restore their recreational use status. The TAT is comprised of representatives from JEA, the Florida Department of Environmental Protection (DEP; Northeast District Office), the City of Jacksonville Environmental Quality Division (COJ EQD), the City of Jacksonville Public Works Department (COJ PWD), the Duval County Health Department (DCHD), the current and former St. Johns River Keepers, the Water and Sewer Expansion Authority (WSEA) and the University of South Florida (USF). The project objective was to develop and evaluate an approach for assessing the source(s) of fecal pollution in the WBIDs of Duval County and to develop a manual for conducting site assessments and evaluating potential sources of fecal contamination to water bodies.

Structure. This paper presents background information on microbial water quality assessment and microbial source tracking, which was used to evaluate potential sources of fecal contamination to the St. Johns River. The methodology used for site evaluations and MST is given, followed by the results of microbial analysis. The discussion places the findings within the context of other recent MST studies, and details the suggested corrective actions presented to the TAT.

Background. Indicator organisms (i.e., fecal coliforms, *Escherichia coli*, and *Enterococcus* spp.) are used as tools to assess fecal contamination of water bodies. They are considered surrogates for human pathogens that are transmitted by the fecal-oral route, including *Salmonella, Cryptosporidium* and enteric viruses. Indicator organism concentrations are monitored in drinking, shellfishing and recreational waters in order to protect public health; however, such measures do not identify the source(s) of indicator organisms. Because knowledge of contamination source is necessary for risk assessment and improvement of the quality of impaired waters, a suite of methodologies known as microbial source tracking (MST) has been developed. MST is an emerging field of microbiology which is focused on linking the fecal microorganisms found in water to their host source (10, 11, 23). A decision-tree or tiered approach relied on a “toolbox” of MST methods, in combination with an extensive collection and mapping of infrastructure (e.g. sanitary sewer overflows, central sewer lines, failure areas for onsite wastewater treatment systems, stormwater infrastructure), historical monitoring and land-use data. These efforts provided the information needed for a weight-of-evidence assessment of the contribution of various potential sources to indicator organism concentrations within these waters. The TAT tested and field-verified the methodology through site assessments of seven selected tributary water bodies (including a relatively unimpacted, control tributary utilized to determine the accuracy of the assessment techniques) and revised the *Tributary Pollution Assessment Manual* based on lessons learned from this

process. In addition to designing a blueprint for conducting site assessments, the TAT identified appropriate corrective actions that could be implemented to reduce fecal coliform levels, with the goal of restoring water quality in the WBIDs to support their designated recreational use. The project's findings and resulting Manual is currently serving as an integral part of the Basin Management Action Plan (BMAP), designed to support the total maximum daily load (TMDL) for fecal coliforms established by the State, for these WBIDs.

METHODS

Preliminary Site Evaluations. Existing databases, obtained from the various agencies, were used in conjunction with land use classifications obtained from the St. Johns River Water Management District (SJRWMD) to delineate WBID categories. These categories were intended to focus source assessment efforts and essentially constituted a pre-assessment based on the best available existing data. The data obtained as part of this effort were used to determine whether a putative source(s) of fecal coliform contamination could be identified for each WBID. A 'putative' source included at least one of the following characteristics: 1) Repetitive, recent and localized Sanitary Sewer Overflows (SSOs) that intersected surface waters, 2) a septic nuisance and/or failure area adjacent to, or intersecting, a waterbody, or 3) targeted land use (e.g., cattle farms, aquaculture, marinas). Therefore, the WBID categories were as follows (see Site-Specific Results for specific WBIDs):

1a. Urban – Putative Source
1b. Urban – No Putative Source
2a. Suburban – Putative Source
2b. Suburban – No Putative Source
3a. Rural – Putative Source
3b. Rural – No Putative Source

One WBID from each category was selected for evaluation (ground-truthing of the draft *Tributary Pollution Assessment Manual*). A map of the sites is shown in Figure 1. The selection process was based on an effort to include those WBIDs that: 1) posed a significant public health issue based on elevated IO levels; 2) geographically represented all of Duval County; and 3) were not undergoing reconstruction that could have impacted the assessment. Thomas Creek was selected as a control WBID (relatively unimpacted by human activities) and was used to verify that analytical methods did not yield false-positive indication of human pollution sources. Three sites per WBID were consistently monitored. The most upstream was labeled -1, and the most downstream was labeled -3. These original stations were supplemented throughout the duration of the project by both temporary and permanent stations sampled by the DEP and the COJ. The supplemental stations were determined by the DEP in conjunction with the COJ and PBS&J and added to the sampling program on an as-needed basis for a variety of reasons: 1) to represent branches of the tributaries that had not yet been sampled; 2) to focus intensive sampling efforts in areas of suspected sources; 3) to test for specific potential illicit discharges.

Sampling Methodology. Each WBID was sampled monthly from July 2005 to February 2006 to integrate both the local wet and dry seasons. Whenever possible, rain

events were captured within the sampling program. If elevated numbers of fecal coliforms were found, more advanced source-tracking techniques were used to identify a specific source-type (i.e., human or ruminant). Sediment samples were taken at sampling stations to assist in the source-identification process. Additional field data such as salinity, pH, conductivity, temperature, turbidity, and dissolved oxygen, as well as general observations, including weather conditions and general tributary characteristics, were also taken to help determine sources of fecal contamination. The entire decision-tree described in the Tributary Pollution Assessment Manual (2006) was implemented, as finances allowed, after each sampling event (Figure 2).

Fluorometry. A flow-through fluorometer (Turner AU-10) was employed to assess the presence of optical brighteners in the tributaries. Samples were processed at Mote Marine Lab, Sarasota, FL. The instrument can be used either from a boat to measure a continuous flow of water on site, or set up in a laboratory and used to measure discrete samples by a cuvette system. Water samples were analyzed with the oil, long wavelength filter kit. The optical configuration included excitation at 300 to 400 nm and emission at 410 to 600 nm.

Enumeration of Indicator Organisms. Water and sediment samples were processed by membrane filtration (0.45 μm pore-size, 47 mm diameter) in order to enumerate fecal coliforms, *E. coli*, and enterococci. Sediment samples were first diluted 1:10 with sterile buffered water and sonicated (2), to release bacteria attached to particles. Fecal coliforms were enumerated on mFC agar after 24 h incubation at 44.5° C (1); enterococci were enumerated on mEI agar at 41°C after 24 h incubation (19); *E. coli* was enumerated on mTEC media at 35°C for 2 h, followed by 22 h incubation at 44.5 °C (20). Colonies on plates were counted and concentrations were reported as CFU · 100 ml^{-1} or CFU · 100 g^{-1} (wet weight) for water and sediment samples, respectively.

Library-independent MST

esp **gene.** Minor modifications of a previously published procedure were used to detect the *esp* gene of *Ent. faecium* (17) by polymerase chain reaction (PCR). Three hundred ml of water and 25 ml of sediment suspension were processed by membrane filtration and incubated on mEI media at 41°C for 48 h. Filters were transferred to 5.0 ml azide dextrose broth (Difco Laboratories, Detroit MI) in sterile, 15 ml conical screw-cap tubes, vortexed vigorously and incubated for 3 h at 41ºC with shaking in order to resuspend cells. Two ml of azide dextrose broth were transferred to a sterile centrifuge tube and centrifuged at 7,500 × g for 10 min. DNA extraction was performed using the QIAamp DNA Stool Mini Kit according to manufacturer's instructions (Qiagen, Inc. Valencia, CA). The forward primer specific to the *Ent. faecium esp* gene, the conserved reverse primer, constituents of the PCR reaction mixture and amplification conditions used were previously published (17). The resulting PCR product (680 bp) was visualized by agarose electrophoresis (1.5% agarose gel) and the image was captured with a FOTO/Analyst Archiver (Fotodyne, Hartland, WI). The positive control for the PCR assay was *Ent. faecium* strain C68. Furthermore, it was shown that each of the PCR reactions were not inhibited in an environmental water and sediment by spiking the samples with *Ent. faecium* strain C68, the positive control for the PCR assay.

Bacteroides. A previously published protocol with minor adjustments was used to concentrate bacterial cells from water for the human and ruminant *Bacteroides* assays

(13). Sixty ml of water, was filtered through a syringe filter (0.2µm pore size, 32mm diameter), followed by back flushing with 1 ml 10 mM Tris (pH 8.0) into a 2 ml microcentrifuge tube. The filtrate was centrifuged at 10,000 rpm for 10 min to concentrate the cells. Following centrifugation, 0.9 ml of the supernatant was removed, and the pellet resuspended in the remaining 0.1 ml. This suspension was used as a template for the PCR. Human and/or ruminant specific *Bacteroides* primers targeting 16S rRNA gene, as well as constituents of the PCR mixture and the amplification conditions have been previously published (13). The resulting PCR product (126 bp) was visualized by agarose electrophoresis (1% agarose gel). In order to assess environmental inhibition, all samples were spiked with the control plasmids as previously described (13). In cases where inhibition occurred, DNA was extracted using QIAamp DNA Stool Mini Kit according to manufacturer's instructions (Qiagen, Inc. Valencia, CA) followed by PCR and gel electrophoresis.

Enterococcus faecalis. A previously published whole-cell PCR procedure that did not require DNA extraction was used for the molecular identification of *Enterococcus faecalis* (9). Following appropriate incubation conditions as described above for mEI, well-isolated colonies were transferred with sterile toothpicks to Enterococcosel broth (180 µl) in individual wells of a microtitre dish, and were incubated at 37º C for 24 h. Cultures exhibiting esculin hydrolysis were streaked on TSA and incubated at 37ºC for 24 h to allow separation of individual colonies. One isolated colony from each culture was transferred with a sterile toothpick to the PCR mix in 0.2 ml microcentrifuge tubes. The constituents of the PCR mix, as well as amplification conditions were previously published (6). The resulting PCR product (941 bp) was visualized by agarose electrophoresis (1% agarose gel). *Enterococcus faecalis* (ATCC 19433) was used as a positive control for the assay.

Library-dependent MST.

The procedure described above for isolation and confirmation of enterococci (mEI, followed by Enterococcosel broth and plating on TSA) was followed. A single colony from each culture grown on TSA was transferred to 1.75 ml of brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) and incubated at 37ºC for 24 h. Genomic DNA was extracted using QIAamp DNA Stool Mini Kit according to manufacturer's instructions (Qiagen, Inc. Valencia, CA). A previously published BOX-PCR protocol utilizing the BOXA2R primer (14), was slightly modified. The master mix contained 5 µl of 5× Gitschier Buffer [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-mercaptoethanol], 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. The PCR amplification conditions used were according to Malathum et al. (14). The PCR product was visualized on a 1.5% agarose gel stained with ethidium bromide, and the image was captured using FOTO/Analyst Archiver (Fotodyne, Hartland, WI).

RESULTS AND DISCUSSION

The results of IO measurements over the 8-month study period demonstrated that each of the 6 tributaries commonly exceeded both the EPA (18) and Florida standard for *Enterococcus* (>104 CFU•100ml^{-1} is considered "Poor" water quality; http://esetappsdoh.doh.state.fl.us/irm00beachwater/terms.htm) and the State standard for fecal coliforms (>400 CFU•100ml^{-1} is considered "Poor" water quality)(7) (Figure 3). Thomas Creek, the tributary designated as the control, was chosen because it was located outside the general influence of sewage and septic systems and was not significantly affected by agriculture. Bacterial levels in this tributary regularly exceeded the Florida State standard as well as the EPA standard, though to lesser degree than the other water bodies. This is likely a result of stormwater contamination, or because natural waters in Florida can support relatively high concentrations of IOs. Bacterial levels in sediments (Figure 4) were generally comparable to those found in the water column.

The original decision-tree methodology was revised to reflect what was discovered to be the most useful assemblage of assessment techniques and sequence. Fluorometry, included as a preliminary technique in the original matrix, has been shown to be a cost effective and reliable technique for identifying domestic wastewater contamination when used in conjunction with IO concentrations (5, 16, 22); however, the methodology presents several logistical constraints when used in shallow, back-water streams (3). One of its primary advantages is that it can provide continuous sampling outputs and patterns of fluorescence, particularly when utilized as a flow-through, vessel-mounted instrument; however, this method is restricted to readily navigable waterways, even when using shallow-draft vessels. Furthermore, in an estuarine environment naturally-occurring substances, such as humic acids or tannins also fluoresce, demanding an extensive post-processing effort to separate the various fluorescence signals (5). Due to the non-navigable nature of the tributaries assessed in this project, fluorometry was concluded to be an ineffective method for the investigation of fecal coliform contamination.

Another change in the original tiered approach was the way in which the *Enterococcus* library-dependent MST approach was used. Originally, this technique was to be used to match genomic profiles of randomly selected enterococci isolated from water and typed by BOX-PCR, to a library of *Enterococcus* types isolated from known fecal sources. One such library developed by Dr. Valerie J. Harwood (University of South Florida) and funded by the FDEP for the Jacksonville region in 2004, included genomic profiles for sources such as sewage, dogs, ducks, wild animals and sediments. Instead of using this technique to identify particular sources, it was used to compare patterns within the genomic profiles of the sediments with those of the water column. Results indicated whether elevated IO concentrations originated from existing sediment reservoirs or whether they originated from more recent and ongoing sources. The method was particularly useful in examining Terrapin Creek where an old cattle farm, currently disconnected from the tributary, was the putative source of bacterial contamination.

BOX-PCR patterns of enterococci isolated from sediment samples collected at Terrapin Creek were more "clonal" (more were identical to each other) than isolates from the corresponding water column, indicating a potential sediment reservoir (Panel A, Figure 5). Twelve of the 20 Terrapin Creek sediment isolates were grouped within one

clade (or family) in which all patterns were 60% similar or greater. In contrast, when this test was exercised at Butcher Pen, where an ongoing source was suspected, the degree of variability in sediment patterns mirrored those of the surface waters, suggesting that continuous inputs were driving fecal coliform concentrations (Panel C, Figure 5). Only four of 19 Butcher Pen sediment isolates fell into the largest clade of >60% similarity. Calculation of the Shannon-Weiner diversity index for these sites, which takes into account the total number of patterns and the frequency of their occurrence, supported this conclusion. A relatively low diversity (many clones) population with a diversity index of 1.34 was sampled from Terrapin Creek sediments, while index of sediment isolates from Butcher Pen 2.36 (relatively high diversity; few clones). Patterns of water column isolates from both sites tended to be much less clonal than those from Terrapin Creek sediments.

The decision-tree primarily incorporates tests which are based on the presence or absence of a particular signal. One exception is the PCR test for *Enterococcus faecalis*. Approximately 50 percent of *Enterococcus* sp. that are isolated from most human sewage samples are *Ent. faecalis*. As a result, a positive presence of *E. faecalis* in a sample does not necessarily indicate a human source, but can be an indicator of human sewage presence. Using the results of this test from Thomas Creek to set a cut-off value, a human signal was defined as being 40% positive for *E. faecalis* for each station at each sampling event. A high percentage of *Ent. faecalis*, combined with additional supporting evidence (i.e., supplementary human-specific test results or evidence gained through site-specific surveys), was considered indicative of the presence of human sewage.

Given the above explanation of the general results obtained through the use of the tiered approach, the findings, specific to each tributary, are described below. It became increasingly apparent during the program that the necessary assessment techniques were becoming progressively more site-specific and needed to be selected based on individual site characteristics, prior results, and knowledge of remaining funds. This considered, all suspicious surface waters or potential illicit connections were sampled and analyzed accordingly. In addition to the information described, it must be noted that stormwater is a potential and highly probable conveyance system for each of these more specific sources. As a result, it must be considered as a source unto itself in order to manage it more effectively.

Site-Specific Results

All statistical comparisons of indicator bacteria concentrations detailed below concern water column samples, unless otherwise specified. Figure 6 shows the frequency of detection for each marker of human sewage at each site (note that all sites were not tested for human markers at each sampling event).

Miramar Creek. Miramar Creek was the WBID evaluated in the Urban, Putative Source category. The results of the assessment techniques utilized as part of the tiered approach strongly indicated that a human source was responsible for the somewhat episodic, but extremely elevated levels of fecal coliform contamination at Miramar Creek. Statistical analysis indicated that mean concentration of fecal coliforms in the water column at sites MR-1 (3.1 $\log_{10}$ CFU/100 ml) and MR-2 (3.6 $\log_{10}$ CFU/100 ml) was significantly higher than at Thomas Creek sites TC-1 (2.9 $\log_{10}$ CFU/100 ml) and TC-3 (2.4 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.05$). A similar relationship was observed in the mean concentration of enterococci where sites MR-1(3.0 $\log_{10}$ CFU/100 ml) and

MR-2 (3.3 $\log_{10}$ CFU/100 ml) were significantly higher compared to TC-1 (2.7 $\log_{10}$ CFU/100 ml), TC-2 (2.3 $\log_{10}$ CFU/100 ml), and TC-3 (1.9 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.01$-0.0001). The analyses of library independent MST indicated multiple positive *esp* tests spread out over the two sampling stations; *Ent. faecalis* was present to a lesser degree (Figure 3 and Figure 6). Due to the urbanized area surrounding Miramar Creek and the low likelihood of significant ruminant sources, the test for ruminant *Bacteroides* was only conducted on one occasion and provided a negative result. Analysis of the sediments indicated consistently high loads of the three indicator species at two stations. Analysis of water samples obtained from the black septic tank discharge and surrounding areas during the field reconnaissance conducted by the COJ in the October, 2005 indicated high concentrations of fecal coliforms. This data aided in conclusion that the principal source of fecal coliform contamination impacting Miramar Creek was malfunctioning septic systems located in this upstream area of the tributary.

Butcher Pen Creek. Butcher Pen was the WBID evaluated in the Urban, No Putative Source category. Concentrations of all IOs were relatively high for the duration of the project with frequent exceedances of the state regulations, particularly at BP-3. Most notably, mean concentration of fecal coliforms in the water column at site BP-3 (3.3 $\log_{10}$ CFU/100 ml) was significantly higher compared to DB-2 (3.3 $\log_{10}$ CFU/100 ml), TC-1 (2.9 $\log_{10}$ CFU/100 ml) TC-2 (2.3 $\log_{10}$ CFU/100 ml), and TC-3 (2.4 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$). Comparably, the mean concentration of *E. coli* found at site BP-3 (3.7 $\log_{10}$ CFU/100 ml) was significantly higher compared to DB-2 (3.1 $\log_{10}$ CFU/100 ml), MR-3 (2.7 $\log_{10}$ CFU/100 ml), TC-1 (2.7 $\log_{10}$ CFU/100 ml), TC-2 (2.4 $\log_{10}$ CFU/100 ml), and TC-3 (2.3 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$). A similar trend was observed in the mean concentration of enterococci where site BP-3 (3.8 $\log_{10}$ CFU/100 ml) was significantly higher than DB-2 (3.4 $\log_{10}$ CFU/100 ml), MR-3 (3.0 $\log_{10}$ CFU/100 ml), TC-1 (2.7 $\log_{10}$ CFU/100 ml), TC-2 (2.3 $\log_{10}$ CFU/100 ml), and TC-3 (1.9 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$). The results of the assessment techniques used at Butcher Pen Creek indicated that a human source was primarily responsible for the elevated levels of fecal coliform contamination (Figure 3 and Figure 6). Comparison of the BOX-PCR patterns of enterococci from the sediments with those of overlying surface waters showed relatively high diversity in water and sediments (Figure 5C and 5D), suggesting recent (ongoing) fecal contamination, in contrast to the results at Terrapin Creek. Analysis of the sediments indicated consistently high loads of the three indicator species at each of the stations sampled. Due to the results of bacteriological assays and the abundance of sewer infrastructure it appears most probable that the source of contamination in this area is sewer-related and stems from the upstream area of the WBID. The test for ruminant *Bacteroides* yielded positive results on only one occasion (Figure 6). Subsequent testing did not detect the ruminant marker, and it is suspected that the ruminant source was a stray deer. Such a sporadic source could not account for the consistently elevated levels of fecal coliform contamination.

Deep Bottom Creek. Deep Bottom Creek represented the Suburban, Putative Source category. Unlike Miramar Creek and Butcher Pen Creek, the results of the assessment techniques used at Deep Bottom Creek did not reveal an obvious source of fecal coliform contamination for this tributary. Analysis of the fecal coliform mean concentrations revealed that site DB-1 (3.1 $\log_{10}$ CFU/100 ml) had significantly higher concentrations compared to TC-3(2.4 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$), and other Thomas

Creek sites to a lesser degree. Examination of the *E. coli* mean concentration showed the same trend where site DB-1 (2.7 $\log_{10}$ CFU/100 ml) had significantly higher concentration compared to TC-3 (2.3 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.05$). Enterococci mean concentrations at Deep Bottom Creek were generally higher compared to the Thomas Creek. Specifically, DB-1 (3.6 $\log_{10}$ CFU/100 ml) compared to TC-1 (2.7 $\log_{10}$ CFU/100 ml), TC-2 (2.3 $\log_{10}$ CFU/100 ml), and TC-3 (1.9 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$).

A human source was indicated by the results of PCR for *Ent. faecalis* at one of the supplemental sampling stations and one detection of *esp* at a mid-stream station; the ruminant *Bacteroides* marker was not detected (Figure 3 and Figure 6). Analysis of the sediments within Deep Bottom Creek indicated high loads of all three indicator species at both upstream and downstream locations.

The evidence indicates that human source(s) are primarily responsible for the elevated levels of contamination in this tributary. A handful of individual systems, possibly including both sewer and septic infrastructure, may be responsible. It appears likely that the actual sources are localized but their positions are dispersed throughout the basin and are episodic in nature, making them difficult to identify when sampled monthly.

New Castle Creek. New Castle Creek was the WBID evaluated in the Suburban, No Putative Source category. Due to the positive results for *Ent. faecalis*, *esp*, and human *Bacteroides*, it was concluded that a human source was contributing to elevated levels of fecal coliform contamination at New Castle Creek. Analysis of sediments within New Castle Creek indicated high loads of the three indicator species at down- and mid-stream stations (the upstream portion of the tributary is channelized and did not allow for sediment samples). Levels of fecal coliforms were highly variable throughout the sampling regime, indicating that the source at New Castle Creek was episodic in nature; however, it is possible that the tributary was impacted by more constant sources but was filling and discharging at irregular intervals. More importantly, extreme levels of fecal coliforms (over 5,000 CFU/100 ml) were only observed on two occasions, both at station mid-stream locations. Mean fecal coliform concentrations at NC-1 (2.5 $\log_{10}$ CFU/100 ml), NC-2 (2.5 $\log_{10}$ CFU/100 ml), and NC-3 (2.6 $\log_{10}$ CFU/100 ml) were significantly higher than BP-3 (3.3 $\log_{10}$ CFU/100 ml) and TC-1 (2.9 $\log_{10}$ CFU/100 ml) (and other Thomas Creek sites) (ANOVA, $P < 0.001$), respectively. The mean concentration of *E. coli* followed the same general trend where NC-1 (2.3 $\log_{10}$ CFU/100 ml) was significantly higher than BP-3 (3.7 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.05$), while NC-2 (2.3 $\log_{10}$ CFU/100 ml) was higher compared to TC-1 (2.7 $\log_{10}$ CFU/100 ml), and TC-3 (2.3 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.05$). Mean concentrations of enterococci were generally higher at New Castle Creek, compared to Butcher Pen, Deep Bottom, and Thomas Creek (*P* value range 0.05-0.0001).

The human-specific markers also indicate that human fecal contamination impacts this WBID (Figure 3 and Figure 6). Because relatively little sewer infrastructure exists in this area, it is probable that contamination is related to improperly functioning septic systems. Although it is possible that the source is sewer-related, the most southern portion of the New Castle WBID encompasses part of a WSEA/DOH-designated septic tank Failure Area which may contribute to contamination.

Terrapin Creek. The Rural, Putative Source category was represented by Terrapin Creek. The putative source was represented by the dairy farm, once located in the northern portion of the Terrapin Creek WBID, which had been isolated from those surface waters by the construction of a new office park. Waters in the WBID were tested, as originally planned, and the analyses conducted for ruminant *Bacteroides* yielded negative results. Surprisingly, a single test for human *Bacteroides* was positive; however, this was attributed to error in sampling water from Dunn Creek that had been blown into the lower reaches of Terrapin Creek by wind-driven currents. In addition, the levels of indicator species throughout Terrapin Creek were generally low to moderate, with few exceptions. Mean concentration of fecal coliforms at TP-1 (2.7 $\log_{10}$ CFU/100 ml), TP-2 (2.5 $\log_{10}$ CFU/100 ml), and TP-3 (2.5 $\log_{10}$ CFU/100 ml) were significantly lower compared to BP-3 (3.3 $\log_{10}$ CFU/100 ml), DB-2 (3.3 $\log_{10}$ CFU/100 ml), and TP-3 (2.5 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.01$, $P < 0.05$, and $P < 0.0001$, respectively). Mean *E. coli* concentrations were generally lower at Terrapin Creek compared to more impacted WBIDs. More specifically TP-1(2.7 $\log_{10}$ CFU/100 ml) was significantly lower than BP-3 (3.7 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.01$), while TP-3 (1.8 $\log_{10}$ CFU/100 ml) was significantly lower than NC-1, 2, 3 (2.3 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.001$).

Enterococci mean concentration followed the same trend where TP-1(3.1 $\log_{10}$ CFU/100 ml), TP-2(2.6 $\log_{10}$ CFU/100 ml), and TP-3 (3.0 $\log_{10}$ CFU/100 ml) were significantly lower compared to BP-3 (3.8 $\log_{10}$ CFU/100 ml) and DB-2 (3.4 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.0001$). It was also obvious that the sediments were harboring high levels of indicator species at each of the three primary sampling stations. In order to confirm suspicions that the primary source of contamination at Terrapin Creek was a dairy farm that had since been closed and isolated from the boundaries of the WBID, an analysis comparing the genomic profiles of the sediments to those of the overlying waters was conducted. Results indicated that the sediments contained a reservoir of contamination that had not been recently augmented, supporting the contention that the original source of pollution had been severed (Figure 5).

Blockhouse Creek. Blockhouse Creek represented the Rural, No Putative Source category. Unlike the other tributaries described above, the results for Blockhouse Creek identified both human and ruminant sources of contamination. Although the analysis of ruminant *Bacteroides* was positive only once, it is likely, given the degree of "light" agriculture throughout much of the area that these farms were contributing to the contamination of the basin's surface waters. In contrast, a human source was indicated by the results of human *Bacteroides*, *esp*, and PCR for *E. faecalis* at the downstream location (Figure 3 and Figure 6). The level of indicator species in the surface waters were typically moderate (fecal coliforms generally between 800 and 2500 CFU/100ml) and were never extreme. The mean fecal coliform concentration at BH-1 (3.4 $\log_{10}$ CFU/100 ml) was significantly higher compared to TP-3 (2.5 $\log_{10}$ CFU/100 ml), TC-1 (2.9 $\log_{10}$ CFU/100 ml), and TC-3 (2.4 $\log_{10}$ CFU/100 ml) (ANOVA, $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). At sites BH-2 and BH-3, mean fecal coliform concentrations were significantly higher compared to Thomas Creek sites (ANOVA, $P < 0.01$). Mean *E. coli* concentrations at BH-1(3.0 $\log_{10}$ CFU/100 ml), BH-2 (2.4 $\log_{10}$ CFU/100 ml), and BH-3 (2.5 $\log_{10}$ CFU/100 ml) were significantly higher compared to TP-3 (1.8 $\log_{10}$ CFU/100 ml) ANOVA, $P < 0.001$). Mean enterococci concentration at Block House

Creek was in general significantly higher compared to the Thomas Creek (ANOVA, P <0.05-0.001). In addition, mean enterococci concentration at BH-2 (2.9 $\log_{10}$ CFU/100 ml) and BH-3 (2.5 $\log_{10}$ CFU/100 ml) was significantly higher compared to DB-2 (3.4 $\log_{10}$ CFU/100 ml) and DB-2 (3.4 $\log_{10}$ CFU/100 ml) (ANOVA, P <0.001 and P <0.01, respectively).Analysis of the sediments within Blockhouse Creek indicated high loads of the three indicator species at each of the three primary sampling locations.

During the field reconnaissance portion of this investigation, it became apparent that there were several potential illicit discharges to the surface waters of Blockhouse Creek, each of which were immediately reported to the DCHD for further investigation. Given these observations in conjunction with the sampling results, weight-of-evidence leads to the conclusion that the source of contamination is likely comprised of numerous human-related, small-scale points of contamination (potentially including sewer and/or septic infrastructure) coupled with relatively minor amounts of input from neighboring farms.

Thomas Creek. Thomas Creek was selected as a control WBID (relatively unimpacted by human activities) and was used to verify that analytical methods did not yield false-positive indication of specific pollution sources. Although this tributary is not pristine, it was chosen because it was closest to being outside the influence of sewer and septic systems and also not highly impacted by agriculture. The analysis of ruminant *Bacteroides* was positive at all three sites and was not surprising given the degree of "light" agriculture throughout much of the area and the possibility of deer frequenting the area. In contrast, a human source was not indicated by the results of human *Bacteroides, esp,* and PCR for *Ent. faecalis* (Figure 3 and Figure 6). The level of indicator species in the surface waters was typically low (fecal coliforms generally below 200 CFU/100ml with occasional peaks between 800 and 900 CFU/100ml). Analysis of the sediments within Thomas Creek did not indicate a substantial load of the three indicator species.

Background information and field observations, together with information gained through the sampling results, suggest that Thomas Creek was an acceptable control for this project. As indicated by the statistical results described above for the individual creeks, mean concentrations of all IOs were significantly lower at Thomas Creek compared to other WBIDs.

CONCLUSIONS

Suggested Corrective Actions. Results from testing of the six priority WBIDs indicate that the dominant source of fecal coliform contamination in Duval County is human-related. More specifically, the three main source-types (not listed in order of importance) were: 1) onsite wastewater treatment and disposal systems; 2) existing utility infrastructure; and 3) stormwater (as a conveyance system). One exception to this generalization was Terrapin Creek. It was determined that the primary source of fecal coliform contamination at Terrapin Creek was the dairy farm that was once located within the northern boundaries of the associated watershed. Given that the hydrology of that basin has changed, such that any remaining livestock have been isolated by new construction of a road and an office park, that source has been disconnected. Further examination of the site would be necessary to determine whether corrective actions should be implemented to remedy the damages already incurred. Implementation of many of the corrective actions discussed below began a few years prior to this project and the impacts have not been fully realized in all impacted tributaries. Nevertheless, it is

recommended that all of these programs continue with refinements derived from these and subsequent detailed assessments of the area tributaries.

Remediation of Onsite Wastewater Treatment and Disposal Systems. Onsite wastewater treatment and disposal systems, or septic systems, have been identified as a leading source of fecal coliform contamination in Miramar Creek, and possibly to a lesser degree in Deep Bottom Creek, New Castle Creek and Blockhouse Creek. Properly designed, functioning, and maintained septic systems can effectively remove nearly all suspended solids, bio-degradable organic compounds, and fecal coliforms (21); however, old and poorly maintained septic systems, such as those throughout much of the Miramar Creek basin, are a major source of fecal coliform contamination. Few programs exist to address onsite system operation and maintenance which result in failures leading to otherwise preventable costs and risks to public health and water resources (21).

Rehabilitation of Existing Utility Infrastructure. Severe and catastrophic utility infrastructure collapses are rare, but as the infrastructure ages, failures become more likely. It appears that this may be the issue of greatest concern regarding fecal coliform contamination at Butcher Pen Creek, and possibly to a lesser degree, if at all, at Deep Bottom Creek, New Castle Creek, and Blockhouse Creek.

Ongoing rehabilitation of existing utility infrastructure is important in order to help reduce or eliminate sanitary sewer overflows (SSOs). Through proper preventative and predictive maintenance and the identification of problem areas, SSOs can be reduced. Since large-scale replacements of existing systems have large capital costs, it is necessary to routinely conduct investigations including but not limited to infiltration and inflow studies to evaluate the integrity of the infrastructure and use this type of assessment to locate severe problem areas with a high probability of impacts related to the utility.

Stormwater Treatment. As mentioned above, it is important to consider stormwater as a potential and highly probable conveyance system for a variety of more specific source of bacterial contamination. As a result, it must be considered as a source unto itself in order to manage it more effectively. The following corrective actions would be helpful in the remediation of pollution to each of the six tributaries evaluated in this project.

Corrective actions using stormwater treatment benefit a tributary by reducing the sediment, oil and grease, and nutrient and bacteria loadings discharged into the affected water body. When focusing corrective efforts on stormwater treatment, it is important to realize that the reduction of sediment loads to a tributary is critical because such particles frequently adsorb bacterial colonies thereby facilitating their survival, growth and transportation from one area to another (4, 8, 12, 15). All of the above, alone or in combination can be effective in reducing coliform loadings to surface waters; however, each must be evaluated based on the area in question, the cost required, and the level of public acceptance. It is recommended that analysis be conducted on a life-cycle cost basis and factors such as capital costs, operation and maintenance costs and regulatory issues be considered in order to determine the most feasible approach.

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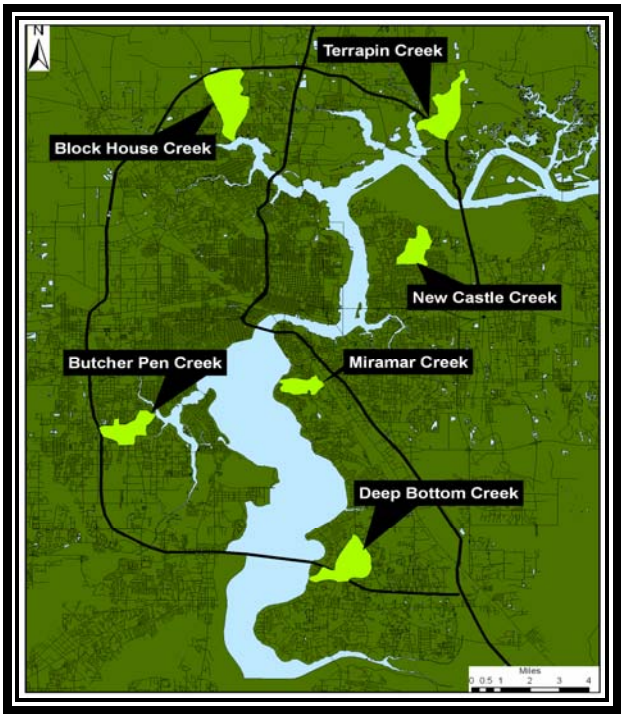

Figure 1. Locations of the tributaries used to field-verify the approach described in the Tributary Pollution Assessment Manual (2006). Thomas Creek is located to the north and is not shown on this map.

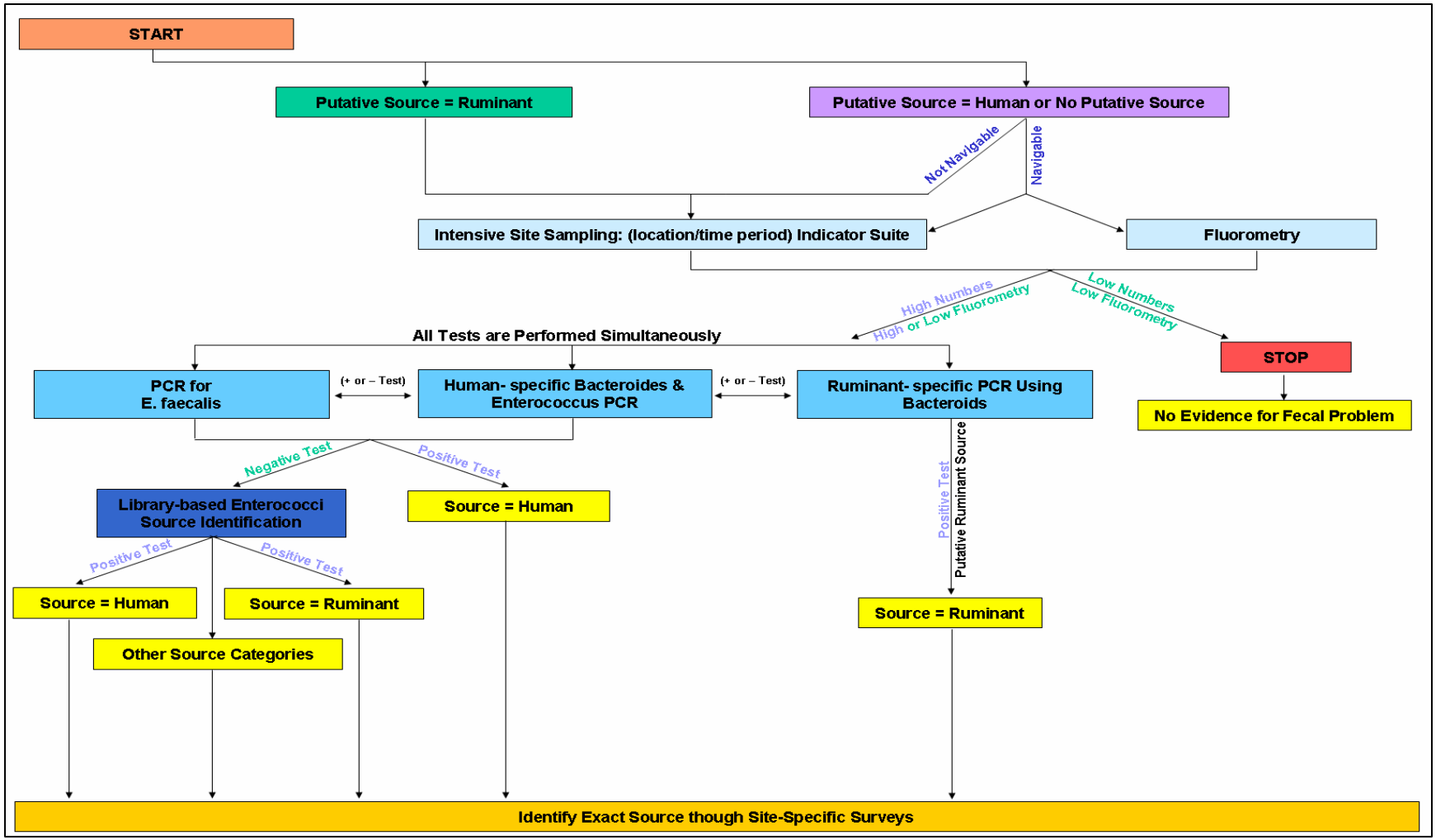

Figure 2. Final decision-tree approach implemented after each sampling event and described in the Tributary Pollution Assessment Manual (2006).

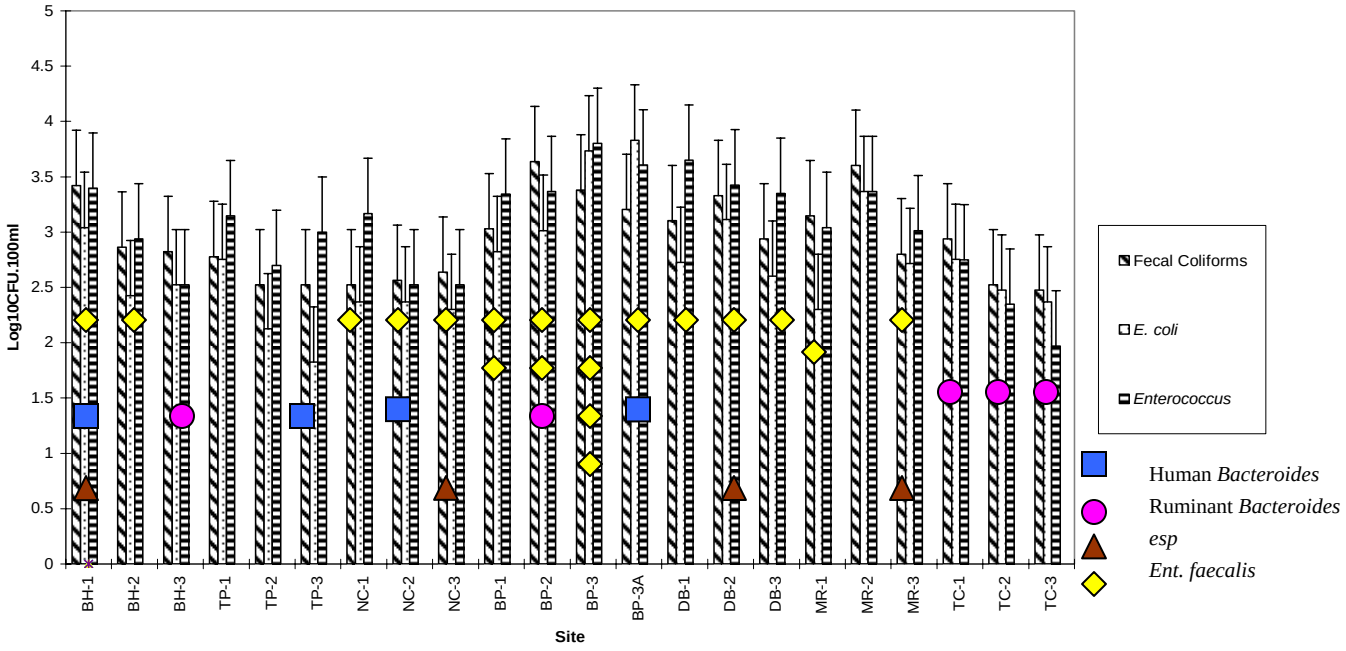

Figure 3. Mean concentrations (log_{10}-transformed) of fecal indicator bacteria in the water column (07/2005-02/2006)

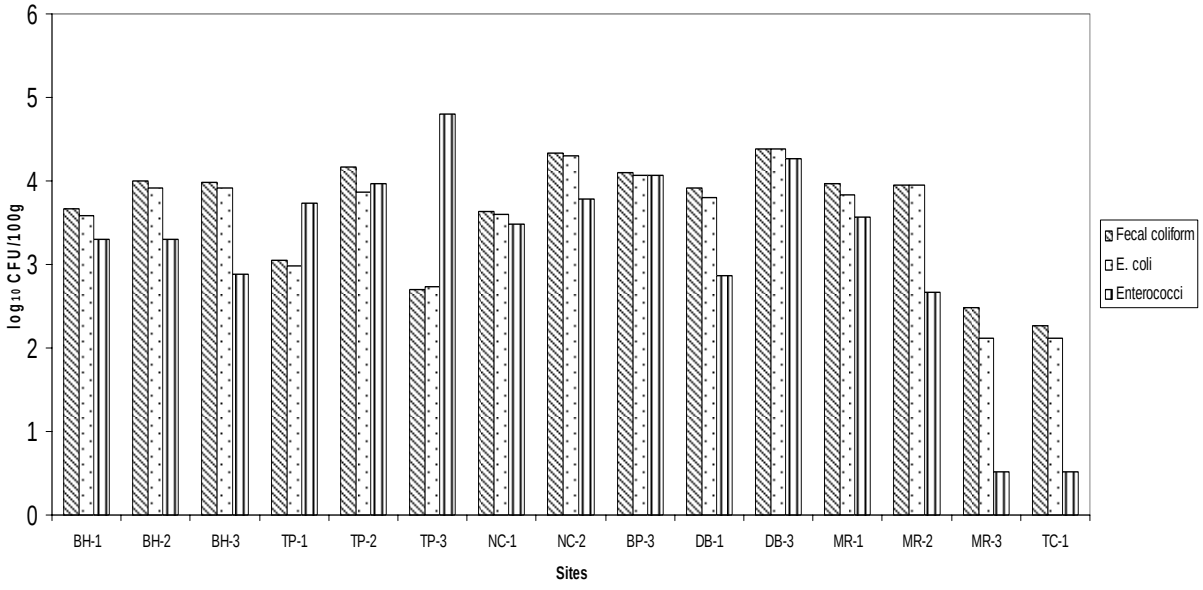

Figure 4. Mean concentrations (log_{10}-transformed) of fecal indicator bacteria in the sediment (07/2005-02/2006)

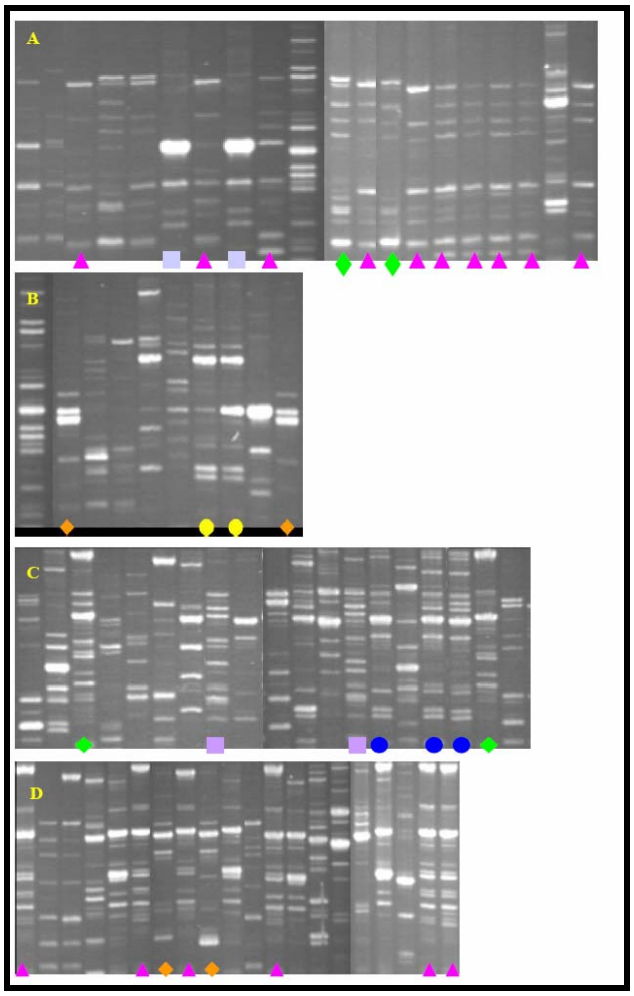

Figure 5. BOX-PCR patterns demonstrating differences in similarity between samples taken from (A) Terrapin Creek sediments, (B) Terrapin Creek water column, (C) Butcher Pen sediments, and (D) Butcher Pen water column. Patterns are read vertically. Within each sample site (A, B, C, or D) those with matching symbols are >90% similar in terms of the banding pattern, and are considered "clones."

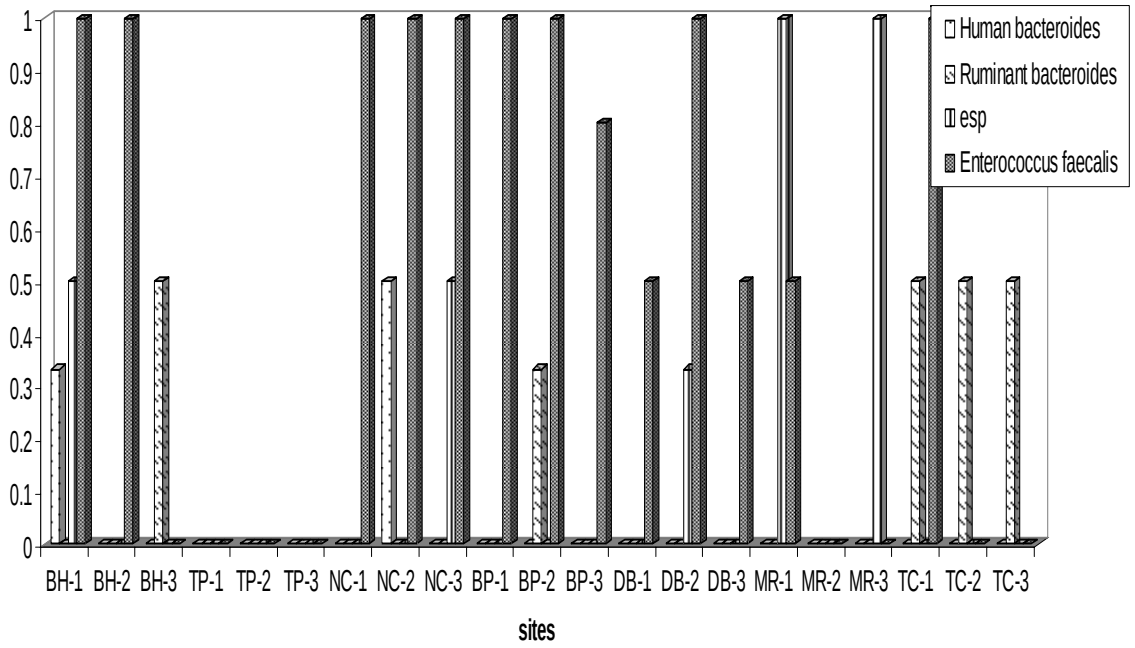

Figure 6. Frequency distribution of human-associated markers: human *Bacteroides,* ruminant *Bacteroides, esp* and *Ent. faecalis.* Note that not all sites were tested at every sample event.

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