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Temporal and Spatial Variability of Fecal Indicator Bacteria: Implications for the Application of MST Methodologies to Differentiate Sources of Fecal Contamination

by

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Abstract

Temporal variability in the gastrointestinal flora of animals impacting water resources with fecal material can be one of the factors producing low source identification rates when applying microbial source tracking (MST) methods. Understanding how bacterial species and genotypes vary over time is highly relevant when the fecal material used to create a source library is collected under very different seasonal conditions than the environmental sample. Our objective was to identify and compare the temporal and spatial variability of fecal indicator bacteria from a specific host in manure and water samples and evaluate the implications of such variability on microbial source tracking approaches and applications. We selected *Enterococcus* as the model fecal indicator, given the supposedly high specificity of some of the species of this genus to the host organism. Cattle was chosen as the model host organism because of the documented high impact that cattle has on impairment of surface waters. The sites studied were located at a farm where cattle have unrestricted access to the stream. Enterococci were isolated monthly from water and manure samples using membrane-*Enterococcus* Indoxyl-β-D-Glucoside agar (mEI) as described in EPA method 1600. The isolates were identified using a multiplex PCR procedure that targets the genus and the species-specific gene superoxide dismutase. Eight species were identified in cattle manure, of which *E. casseliflavus (*37%*), faecium* (22%*)* and *hirae (*18%*)* were the most abundant. Nine species were identified in stream samples with *E. faecalis (*43%*), casseliflavus/flavescens (*34%*), and hirae (*11%*)* being the most abundant. September exhibited the highest species abundance in manure samples while March had the highest species abundance in stream water samples. *E. assini* and *E. malodoratus* were only detected in manure samples, but were not detected in water samples. In contrast, *E. durans, gallinarum and sulfureous* were only isolated from the stream samples. In general, the enterococci distribution pattern and species richness found in manure samples did not correlate with those found in the stream samples at the individual species level. However, cluster analysis revealed strong seasonal and spatial variability of groups of enterococci, and indicated that some clusters that seem specific to manure can be found in the water only during certain seasons. In addition to the enterococci library development, 16S rDNA host-specific *Bacteroides* markers were also applied to the water samples. The results indicate that data obtained with the *Bacteroides* markers (BM) generally agreed with the enterococci data showing higher occurrence of the cattle BM in areas under obvious cattle impact. However, no seasonality was identified in conjunction with any of the BMs used. In addition, the cow marker was also detected at an upstream-of-the-farm location that was not under obvious cattle influence. This study suggests that in order to increase the validity of MST methods, it is necessary to consider temporal variability when designing the sampling scheme of the source material and constructing source libraries, and increase the specificity and field testing of DNA-based markers.

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Temporal and geographic stability of fecal indicator bacteria

An ideal source tracking organism must be stable in the environment. Sampling performed over time should not reveal significant genotypic or phenotypic variability within host individuals or within host populations. In addition, variable environmental conditions such as temperature and pH, and host factors such as quality and type of feed or antibiotic treatments, etc., should not affect an ideal indicator. All these conditions affect the host organism and, in turn, the inside environment that the source indicator bacteria inhabit. To date, very few microbial source tracking (MST) studies have addressed the temporal stability of fecal indicators, making it difficult to reliably identify sources over time. In this study, we sampled manure and impacted manure water monthly at a Georgia farm site over a year to determine: the temporal variability of various species of *Enterococcus*; the spatial distribution and stability of enterococci species in stream water; and which species might be the most relevant and promising specific indicators of the host organism, i.e., beef cattle.

Temporal stability

When addressing temporal variability, it seems important to establish the difference between transient and resident populations of source indicators. This is of particular relevance if, for example, the range of clones estimated in natural populations of *Escherichia coli* (100-1000 per host species) (Selander et al. 1987) are found to be comparable for other fecal indicator bacteria. Caugant et al. (1981) defined a transient population as one observed at only one sampling time, while a resident population is observed at more than one sampling time. In order for MST methods to be effective, the source indicator bacteria selected

should be part of the resident population of the source species. In addition, it should be part of a clonal population that is stable through time as suggested by Gordon (2001) for *E.coli*. In a study performed over an 11-month period on a single human host, Caugant et al. (1981) found a significant difference between the resident and the transient populations of *E. coli*. The resident population accounted for only 5.6% of the 53 electrophoretic types identified using multilocus enzyme electrophoresis. Jenkins et al (2003) also found a rather low percentage of *E. coli* ribotypes to be part of the resident population in yearling steers sampled four times over a 129-day period. Specifically, only 8.3% of 240 ribotypes were determined to be resident in the host species. In addition, no ribotype was found at all four sampling times or in all of the steers sampled from a total of 20 resident ribotypes. Also using *E. coli*, Ochman et al. (1983) observed that the resident population from multiple hosts accounted for only 8% of all the electrophoretic types identified, and only 5 types were found in more than 7 hosts. These results suggest that there is a high probability that the majority of ribotypes obtained from a single host species at any given time belong to transient populations. This observation has major repercussions relative to the establishment of host origin libraries, that could require continuous updating in order for a particular MST methodology to be able to track the host species (Jenkins et al. 2003) over an extended period of time.

It should be noted that although a general lack of temporal stability seems to be a big limitation in the identification of suitable source indicators, there are certain source genotypes that have been recovered from environmental samples after extended periods of time. These periods range from a few weeks to a year

(Faith et al. 1996; Jenkins et al.2003; Wiggins et al. 2003). Restriction endonuclease digestion profiles (REDP) performed in dairy cows from 70 farms in Wisconsin revealed that two isolates exhibited the same REDP even though they were sampled 7 months apart. Results from the same study also indicated that a herd or animal can contain isolates of *E. coli* O157:H7 that have multiple, but similar profiles; however, most of these profiles were found to change over time (Faith et al. 1996). Long-term temporal stability has also been observed for some indicator organisms using phenotypic tests such as antibiotic resistance patterns (ARA) (Wiggins et al. 2003).

Geographic stability.

Three main assumptions can be made when investigating the geographical stability of an ideal source indicator. These are that: a) a bacterial source indicator exhibits "geographical structure", that is, the similarity of the bacterial indicator in various populations of a given host animal species is directly proportional to the geographical distance of the members of such population; b) a bacterial source indicator sampled from one population of a given host animal species will be similar to a bacterial source indicator sampled from any other population of the same host animal species, and a predictive relationship can be established between the two; and c) a bacterial host indicator sampled from various populations of a given animal host species separated by great geographic distances exhibits a high similarity index and accurately tracks the host species.

Studies indicate that the first assumption regarding "geographical structure" for populations of the same host animal species is hard to verify for

human hosts. This is attributed to the mobility of humans among geographic areas. On the other hand, isolates from non-domesticated animals seem to exhibit more "geographic structure" due to their restricted movement patterns (Gordon 2001). Caugant et al. (1984) reported that "geographic structure" was hard to demonstrate for *E. coli* in families living within the same city, where only 6% of the diversity was explained by the geographical separation, and 1% of the diversity was explained by the distance separating families living in different cities. Another possible factor affecting "geographic stability or structure" is that the host animal digestive system can select for particular resident bacterial strains, generating a very specific gut flora in each host (Souza et al. 2002), making it difficult to identify genotypes and/or phenotypes over broad geographic areas.

An important consideration when trying to assess spatial stability is the analysis methodology used. In a study performed across a broad geographical area in Florida, researchers used a one-enzyme ribotyping procedure to determine the accuracy of this MST methodology to identify beef and dairy cattle, poultry, swine and human host species using *E. coli* isolates (Scott et al. 2003). Although the methodology was accurate differentiating human vs. non-human hosts, it failed to distinguish among the different non-human host species across the broad geographical region. In contrast, Hartel et al. (2002) were able to successfully apply a two-enzyme ribotyping methodology to discriminate among *E. coli* ribotypes isolated from cattle and horses from two locations (Georgia and Idaho). The results from this study support the first assumption for geographic stability, but do not support the second and third assumptions. The latter

researchers indicated that up to a distance of 260 km, there is ribotype sharing among isolates obtained from horses. Cattle exhibited some ribotype sharing up to a distance of 350 km, but not at a distance of 2900 km (Georgia and Idaho isolates). However, for swine and poultry, the percent sharing was rather low and not significantly different from locations closer together (locations within Georgia) than far apart (Georgia and Idaho). Using a similar ribotyping method, human vs. non-human hosts were also accurately identified from *E. coli* isolates across an extended area in the Apalachicola region of Florida (Parveen et al. 1999).

For library-based methods, the size of the library seems to be a determinative factor supporting the second and third assumptions presented in this section. In a study using ARA, results indicated that merging 6 watershed libraries to encompass a total of 6,500 isolates produced a library large enough to be representative and capable of being used to accurately identify enterococci host species across a broad geographic area (Wiggins et al 2003). These latter study results also suggested that the minimum size of a library should be about 2,300 isolates in order for it to be representative, in this way, it is possible to create multiwatershed databases representative enough for the reliable identification of fecal bacterial sources.

Study Site and Sample Description

Water and manure samples were collected at Chandler Farm (CF), a beef cattle farm located in Madison County, Northeast Georgia (Figure 1).

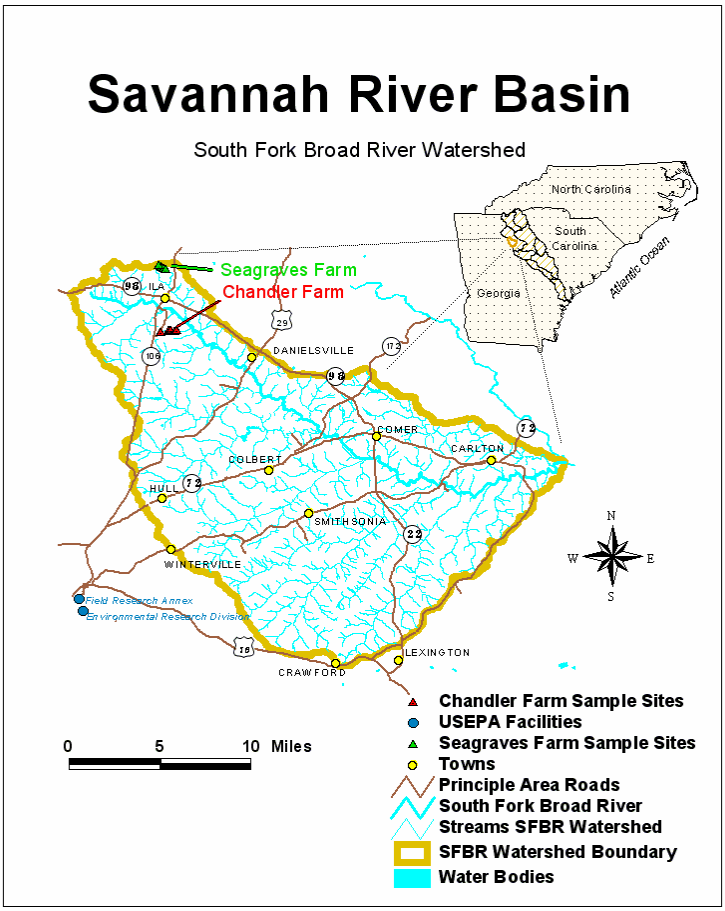
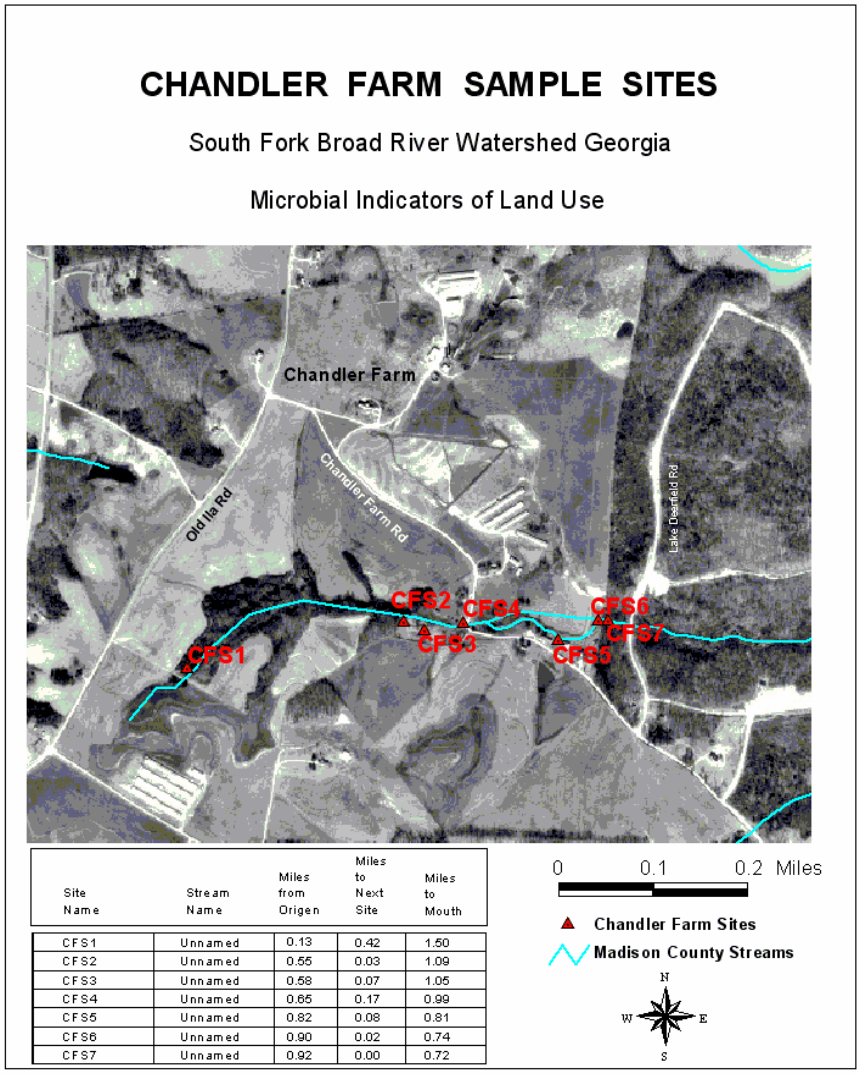


Figure 1: Study site location

Water samples were collected from a first order stream that crosses the farm from west to east and is a tributary of the South Fork Broad River (Figure 2). Seven sampling locations were located along the stream within the Chandler farm site (Table 1) covering a distance of 2.3 km. One liter water samples were collected at each location once per month from September 2003 through January 2005. During each sampling campaign, five fresh cattle manure samples were also collected from different individuals after collecting the water samples.



Site Name	Stream Name	Miles from Origen	Miles to Next Site	Miles to Mouth
CFS1	Unnamed	0.13	0.42	1.50
CFS2	Unnamed	0.55	0.03	1.09
CFS3	Unnamed	0.58	0.07	1.05
CFS4	Unnamed	0.65	0.17	0.99
CFS5	Unnamed	0.82	0.08	0.81
CFS6	Unnamed	0.90	0.02	0.74
CFS7	Unnamed	0.92	0.00	0.72

Figure 2: Sampling locations at Chandler Farm, Georgia.

Table 1: Description of stream water sampling locations at Chandler Farm, Georgia.

Site ID	Description of Site	Direct Cattle Impact on Site	Distance from Origin of Creek (km)
CFS-1	Creek headwaters, upstream from cattle impact	None	0.21
CFS-2	Stream at cattle crossing area	High	0.89
CFS-3	Unrestricted access of cattle to creek	High	0.93
CFS-4	Intermittent unrestricted access of cattle to creek	Medium	1.05
CFS-5	Stream by side of pond, cattle was never observed in this location	Low	1.32
CFS-6	Stream at outlet of pond, cattle was never observed at this location	Low	1.45
CFS-7	Stream outside of property fence, no direct access by cattle	Low	1.48

Methodology

After preparing slurries of the manure samples, both the manure and stream water samples were processed by membrane filtration to obtain the total number of enterococci in the water and to isolate enterococci species for library development. The specific procedure is depicted in Figure 3.

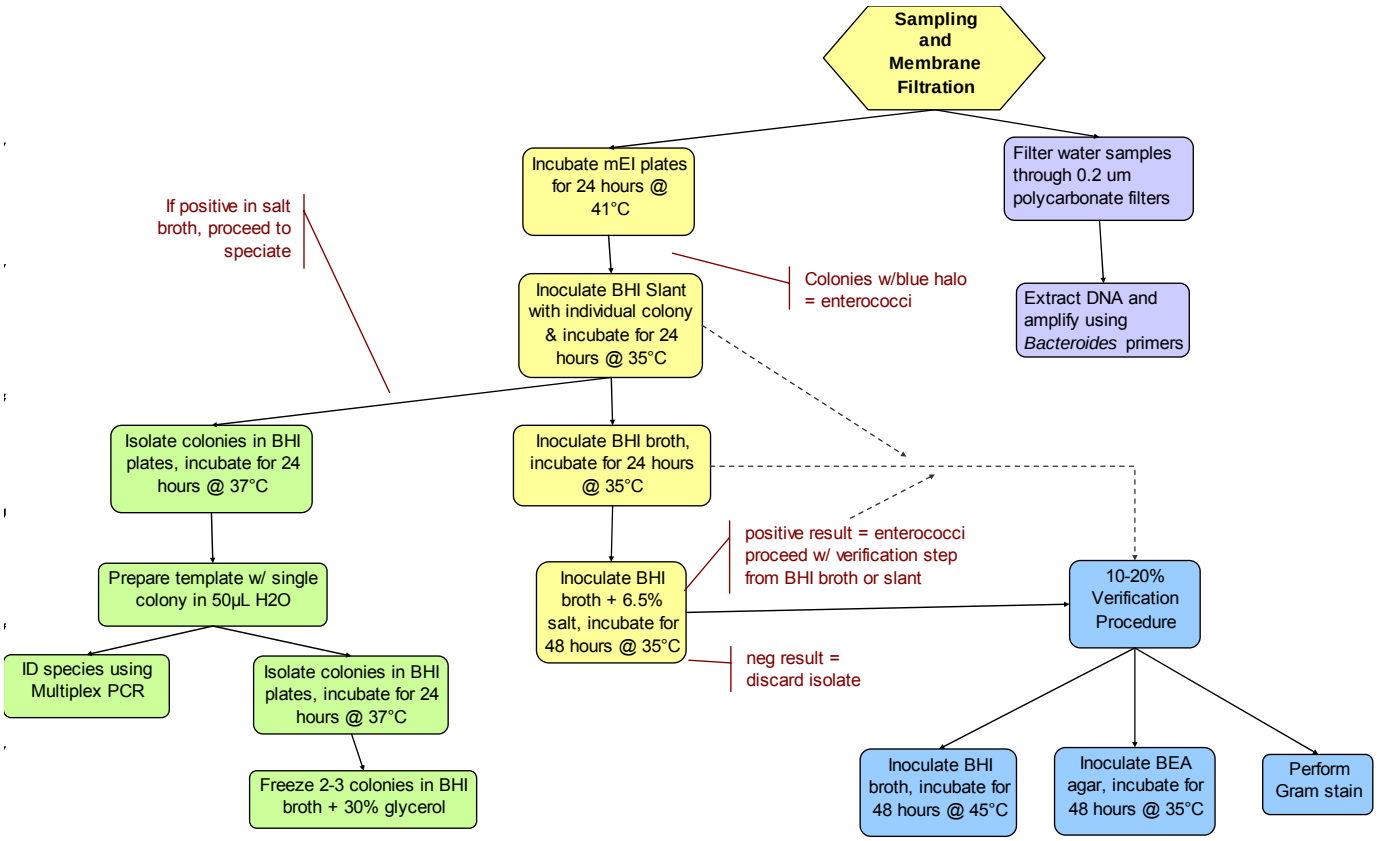


Figure 3: Procedure diagram for counting, isolating, verifying and speciating enterococci in environmental samples.

Procedure for Verification of Enterococci Species

A modification of EPA Method 1600 was used to count, isolate and verify enterococci from the environmental samples. Briefly, 1, 5, 10, and 50 ml of stream water and 10 and 25 ml of a 1 x10^{-6} dilution of manure slurry were filtered through 0.45um cellulose membranes and incubated on membrane-*Enterococcus* Indoxyl –β-D-Glucoside (mEI) agar plates at 41 ± 0.5˚C for 24 hours. After incubation, all colonies having a blue halo were considered to be presumptive enterococci. Five colonies from each location water sample and ten colonies from each manure sample were isolated on brain-heart infusion agar

(BHIA) slants and in a tube of brain-heart infusion broth (BHIB), using a 1ul loop. The BHIA and BHIB samples were then incubated at 35 ± 0.5˚C for 48 and 24 hrs, respectively. After incubation, a loop-full (1ul) from each BHIB tube exhibiting growth was transferred to a tube of BHIB plus 6.5% NaCl, and incubated at 35 ± 0.5˚C for 48 hrs. Any isolate not exhibiting growth on BHIA, BHIB or BHIB + NaCl was considered to be non-enterococci, and was not used any further in the procedure.

About 20% of the isolates exhibiting growth on the media mentioned aboved were further verified as *Enterococcus* using the following procedure: a 1ul loop of sample was taken from a BHIA slant and transferred to a Bile-Esculine Agar (BEA) slant, and a tube of BHIB. The BEA slant was incubated at 35 ± 0.5˚C for 48 hrs and the BHIB tube was incubated at 45 ± 0.5˚C for 48 hrs. Finally, a Gram stain was performed on the final isolates. Growth in each medium combined with and identification of the final isolate as Gram positive cocci verified the isolate as an *Enterococcus*.

Multiplex PCR procedure

All polymerase chain reactions (PCR) were conducted within currently established EPA Quality Assurance/ Quality Control guidelines. The workflow was conducted such that the opportunity for sample contamination was reduced as much as possible. It was imperative that reagent preparation, sample preparation, DNA extraction and PCRs followed a one-directional flow in separate areas with separate pipettes and equipment to prevent cross-contamination. All reagents were prepared in working volumes in a positive pressure room on a clean bench after exposing the bleach-disinfected area to UV

light for 10 minutes. DNA extractions were conducted in a separate laboratory. PCRs were run in a third location, physically separated from the reagent and DNA prep rooms. Pipettes and lab coats were dedicated to the different steps of the procedure. Environmental samples were processed following standard microbiological aseptic techniques. Positive controls for the PCR were used in each run to insure that the PCR was not inhibited by contaminants. Negative controls (reagent blanks) were used to insure that amplified DNAs were only coming from the environmental samples, and not introduced to the samples at the laboratory. PCR optimization for the *Bacteroides* work was performed at the beginning of the study.

Speciation of enterococci isolated from manure and stream water samples was performed as depicted in the lower left side of Figure 3. After verifying the isolates as *Enterococcus*, whole cell templates were prepared in molecular grade sterilized water. These templates were used for up to three weeks. Seven master mixes were used to identify up to 23 species of *Enterococcus* using a multiplex PCR procedure based on the superoxide dismutase gene (Jackson et al., 2004). The procedure was performed testing the isolates with the master mixes in the following order: 1, 2, 6, 4, 3, 5, and 7. The majority of the isolates could be speciated by applying only the first three master mixes in the sequence, thereby achieving the best use of resources and the most time and cost effective approach. PCR products were separated and identified using a 2% 1X TAE agarose gel containing 2 µg/ ml ethidium bromide. Gel analysis was performed using a EpiChemi Darkroom BioImaging System (UVP, Inc.) equipped with a transilluminator, and fitted with Labworks 4.5 software. Band sizes were

identified by comparing the sample DNA to the positive controls included with each run, and by comparing the band size to a 100 bp DNA ladder. Once the isolates were identified, the templates were plated again on BHIA and 3 to 4 single colonies were inoculated into BHIB containing 30% glycerol. The inoculated medium was stored at -80˚C.

DNA Extraction and Amplification with Bacteroides Primers

DNA Extraction from Fecal and Water Samples. Manure fecal samples were stored at -20^{o}C immediately upon arrival at the lab until DNA could be extracted. DNA was extracted with a MoBio UltraClean® fecal DNA mini kit using 0.25 gram of fecal material according to the manufacturer's instructions. Water samples (100 ml aliquots) were filtered through 0.4 µm cellulose filters and DNA was extracted from the membrane filters using a Qiagen DNeasy® tissue kit, following the Qiagen protocol for DNA extraction with a micro-centrifuge and an additional wash of Buffer AW2 (included in the kit).

Amplification using Bacteroides Primers. One general, two cow-specific and two-human specific-*Bacteroides* primers (Bernhard and Field, 2000) were used to test water samples. After applying PCR optimization procedures, the following program was used: initial denaturation at 94˚C for 2 minutes, product amplification by 30 cycles of denaturation at 94˚C for 1 minute, annealing at 53/54˚C for 1 minute, and elongation at 72˚C for 1.5 minute. Amplification was followed by a final extension at 72˚C for 3 minutes. *Bacteroides*- PCR products were identified (presumptive positive result) in a 1% agarose gel containing ethidium bromide by comparing the band intensities under UV light to the intensities of a commercially available 100 bp DNA mass ladder.

Statistical Analysis

A hierarchical cluster analysis was performed (using Minitab v.12 statistical software) on the165 samples taken from cow manure patties, upstream-of-the-farm stream water, and on-the-farm stream water. The objective of this analysis was to group together samples that showed similar relative abundances of the most common species of *Enterococcus*. Enterococci species that were found in only a few of the 165 samples were not included in the analysis. The following five species were seen frequently enough to be included: *E. casseliflavus, E. faecalis, E. faecium, E. flavescence, E. hirae.* In addition, we included one category that was the sum of all unidentified enterococci species.

In the first step of the clustering algorithm, the two samples with the most similar *Enterococcus* species relative abundances are grouped together. These two observations are now designated as a cluster, and this cluster is represented by a centroid, or mean value, of the two samples that compose it. In step two, all remaining samples are examined and the next two that have the most similar relative species abundance are grouped or clustered. In each subsequent step, the two samples (or possibly clusters) that exhibit the greatest similarity are grouped together. Hierarchical clustering requires that a subjective stopping-point be chosen as the algorithm progresses. If this is not done, the algorithm will eventually form one large group of all observations. We stopped the procedure after step 121, prior to the formation of two large clusters. At this point, 15 clusters had been formed with member species (could be the same or different species) appearing 3 or more times in each one of the 165 samples; 134 of the 165 total samples were found within these 15 clusters. The other 31

samples (19% of the total sample pool) were identified as “outliers”, meaning that their enterococci communities did not match well with communities seen in the other samples.

After stopping the algorithm, we recorded the centroid of each cluster (i.e., the mean relative abundances for the five enterococci species used in the analysis) plus the general enterococci classification. The centroids for the 15 clusters with member species that appear 3 or more times are given in Table 5. In our final step, we used the cluster designations for each of the samples to perform ANOVA and MANOVA analyses to test for differences in the clusters found for manure, upstream-of-the-farm stream water, and on-the-farm stream water, as well as changes in the seasonal occurrence of the clusters.

Results and Discussion

Total Enterococci Counts in Stream Water Samples and Comparison of Fluorescent Assay and Membrane Filtration Procedures. As indicated previously, total enterococci counts were performed using EPA method 1600. Accordingly, sample volumes of 1, 5, 10, and 50 ml were used to target total counts into the suggested range of 20 to 66 colonies/100 ml. A defined-substrate assay method from IDEXX laboratories that applies a methyl-umbelliferyl-β-glucuronide (MUG)-based medium (Enterolert®) for detection of enterococci was also used on the water samples. The objective of the test was to perform a comparison of the results obtained from both methodologies and to evaluate their accuracy. Figure 4 shows the total enterococci counts obtained by the membrane filtration procedure (mEI) for the samples collected at the seven water

sampling locations. The results indicate that the highest counts were always obtained at locations CFS-2 thru -4, locations directly impacted by cattle, including a cattle crossing point (CFS-2). Locations CFS-5 thru -7 exhibited one- to two-fold less total counts than the upstream locations (with summer values being slightly higher than those in other seasons), indicating a decrease (due to dilution, settling, dye-off, etc.), of the fecal bacteria in the water column. Although the highest counts were observed mostly from April through September that tend to be months of low precipitation, monthly variability of the counts didn't allow us to establish significant differences.

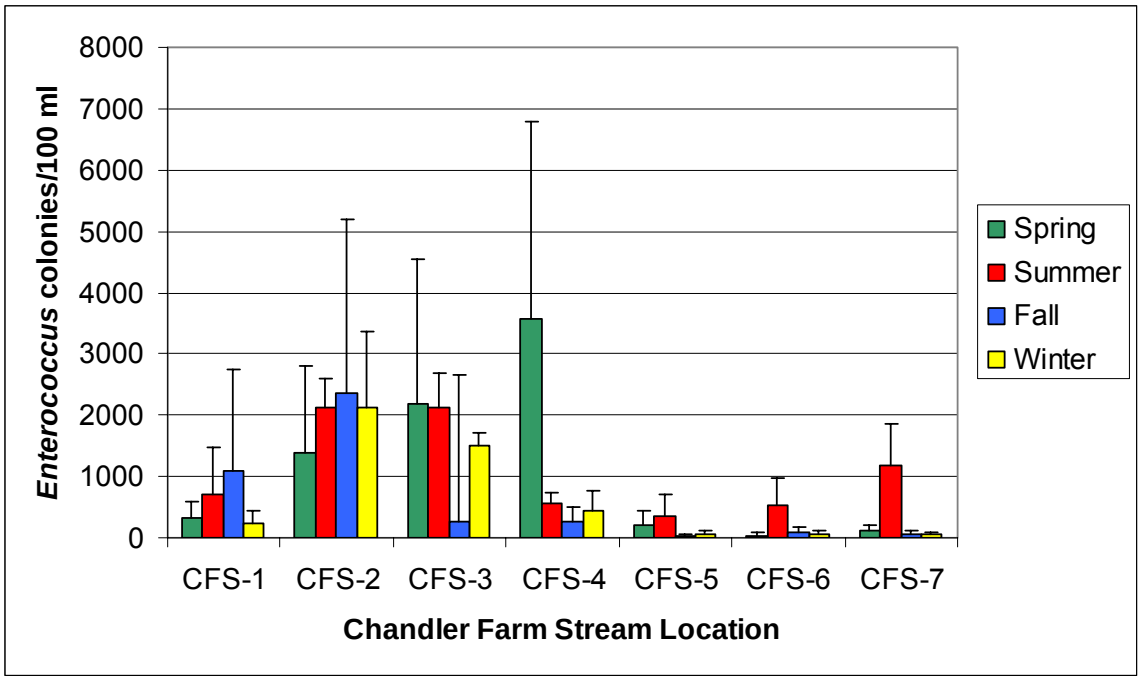


Figure 4: Seasonal enterococci counts in water samples collected at a Georgia cattle farm using the mEI membrane filtration procedure.

The accuracy of the mEI procedure was determined by comparing the number of isolates originally obtained from the mEI plates per site with the number of isolates identified as *Enterococcus* with the multiplex PCR. In mEI,

colonies of any color that produce a blue halo are presumptive enterococci. Our results indicate that in water, an average of 99% of all isolates that produced a blue halo in the mEI, also tested positive in the salt broth (which is one of the biochemical tests run to verify the presence of enterococci). However, this agreement was down to 21% for the manure samples. Those isolates that tested negative in the salt broth were discarded, as a test of them with the multiplex PCR demonstrated that they were not of the genus *Enterococcus.* Of the isolates that tested positive in the salt broth, 99 and 97.5% were identified as enterococci in the water and manure samples, respectively, indicating that salt tolerance was indeed a good indicator for the presence of enterococci in this type of stream water and manure samples. In contrast, the presence of a blue halo in the mEI was not a good indicator of the presence of enterococci in manure samples, since only 43.8% of those isolates with a blue halo were identified as enterococci by the multiplex PCR. For water, however, 97.6% of all those isolates exhibiting a blue halo were identified as enterococci by the PCR procedure (Figure 5).

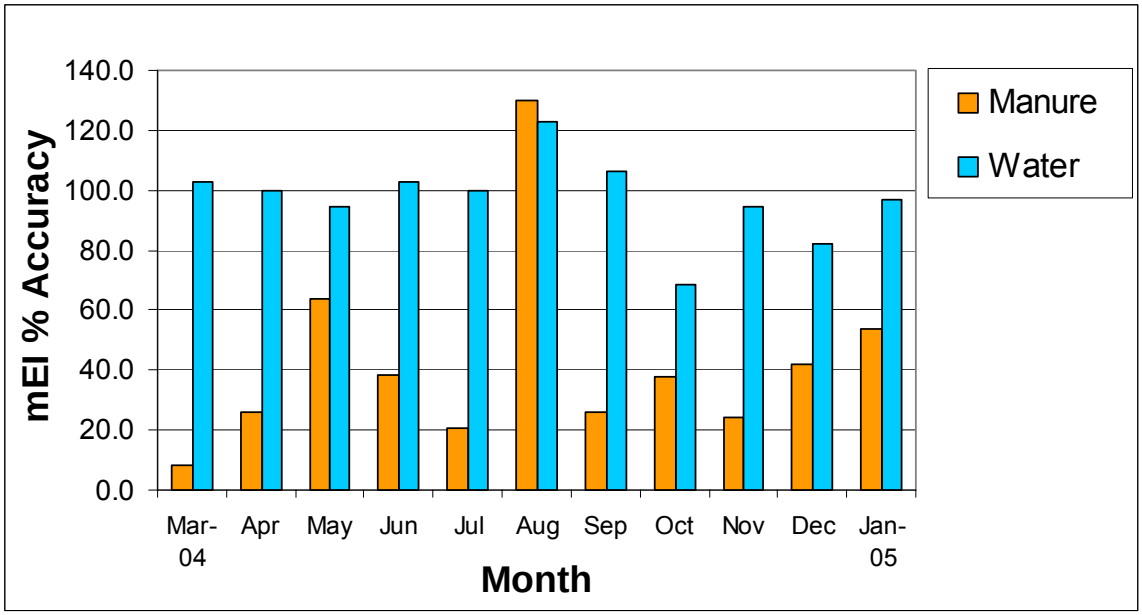


Figure 5: Percent of isolates with a blue halo isolated from mEI that tested positive for the genus *Enterococcus* with a multiplex PCR procedure.

The higher than 100% accuracy indicated for some of the samples in Figure 5 is due to the presence of additional enterococci colonies mixed with isolates believed to be only one type of colony when they were originally isolated from the mEI. The mixed colonies were separated into pure cultures and identified as *Enterococcus* by the multiplex PCR.

We compared the precision of the Enterolert® method relative to the mEI for determination of the total number of enterococci in water samples by calculating the relative percent difference (RPD) of the total counts per 100 ml obtained with each method. The RPD was calculated using the following equation:

RPD= (mEI counts-Enterolert counts/(mEI counts + Enterolert counts)/2) * 100

The calculated results indicated that there is an average of 32 ±13.7% underestimation in the total *Enterococcus* counts obtained with the Enterolert procedure relative to the mEI method for the type of water samples used in this study (data not shown). In addition, no correlation was observed between the two methodologies. Kinzelman et al. (2003) also reported a lack of correlation between these two methods; however, they found that the Enterolert® procedure generated false positive results that produced an overestimation of the actual number of enterococci, contrary to the underestimation found in our study. Therefore, it is possible that the performance of the Enterolert® procedure is highly dependent on the physical/chemical conditions of the environment tested, and probably more studies are necessary to determine its general efficacy in freshwater systems.

Composition and Temporal Variability of *Enterococcus* Species in Manure and Water. A total of 11 species of enterococci were identified in water and manure samples collected during our study using a multiplex PCR procedure (Jackson et al., 2004). *E. malodoratus* and *asini* were only found in manure while *E. sulfureus*, *gallinarum* and *durans* were only found in the water samples (Table 2). Because *E. malodoratus, asini* and *gallinarum* were only found once during the whole sampling period, they were categorized as transient species in the system (Caugant et al., 1981). *E. durans* and *sulfureus* were found in several occasions during different seasons (data not shown) and were

not believed to be transient species, but were not observed frequently enough as to be considered important members of the enterococci community.

Table 2: *Enterococcus* species isolated from cattle manure and stream water at a farm in Georgia.

Enterococcus species	
Manure	Water
E. casseliflavus	*E. casseliflavus*
E. faecalis	*E. faecalis*
E. faecium	*E. faecium*
E. flavescence	*E. flavescence*
E. hirae	*E. hirae*
E. mundii	*E. mundtii*
E. malodoratus	*E. sulfureous*
E. assini	*E. gallinarum*
	E. durans

The % abundances of the most common enterococci species found in manure and water samples are presented in Tables 3 and 4, respectively. We found greater variability in the seasonal abundances of individual species in manure than in water. In manure, *E. faecalis* was the most, while *E. casseliflavus* and *E. flavescens* were the least abundant abundant during spring (Table 3). During summer, *E. casseliflavus* , *E. faecium* and *E. flavescens* were all in high abundance (Table 3). It is clear that the relative % abundance of the individual species in manure varies as a function of season; indeed, the results indicate that *E. hirae* and *E. faecium* were completely absent during summer and winter, respectively. In contrast, it was not possible to identify any clear seasonal trend in the % composition of the different species in water due to the

high degree of seasonal variability observed. For instance, *E. faecalis* was found in high abundance during the fall in both farm and upstream-of the-farm locations. In contrast, during spring, it was found at relatively high abundance only in manure. High seasonal variability was noted in the *Enterococcus* populations isolated from water at the upstream-of-the-farm location, indicating that the background Enterococcus populations (in wildlife and possibly poultry due to the proximity of chicken houses to this site) are as variable as the Enterococcus populations isolated at the farm sites. In general, these results suggest that the five most abundant enterococci species are ubiquitous in the environment, given the fact that they were found in water samples that are not supposed to be impacted by cattle (CFS-1). In addition, the general use of individual species to establish seasonal and/ or source trends is likely to be a difficult task due to the high degree of variability observed.

Table 3: Seasonal % composition (mean ± sd) of the most common *Enterococcus* species isolated from cattle manure samples collected at a cattle farm in Georgia.

Season	E. casseliflavus	E. faecalis	E. faecium	E. flavescens	E. hirae
Spring	4 ± 7*	34 ± 32*	6 ± 14	3 ±7*	22 ± 32
Summer	40 ± 43	4 ± 14	21 ± 32*	28 ± 39*	0
Fall	43 ± 31	12 ± 22	14 ± 24	12 ± 15	13 ± 27
Winter	49 ± 29	4 ± 14	0	12 ± 29	17 ± 27

*Significantly different than winter

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Table 4: Seasonal % composition of the most common *Enterococcus* species isolated from water samples collected in a stream located at a beef cattle farm in Georgia.

Season	Sample location	E. casseliflavus	E. faecalis	E. faecium	E. flavescens	E. hirae
Spring	CFS-1	27 ± 12	60 ± 35	0	0	7 ± 12
Summer	CFS-1	17 ± 17	50 ± 44	6 ± 10	6 ± 10	0
Fall	CFS-1	0	56 ± 38*	7 ± 16	14 ± 27	11 ± 20
Winter	CFS-1	11 ± 19	44 ± 51	0	28 ± 25	8 ± 14
Spring	CFS-2 thru 7	27 ± 27	26 ± 24	7 ± 5	3 ± 10	21 ± 19
Summer	CFS-2 thru 7	27 ± 21	26 ± 21	1 ± 6	29 ± 26	1 ± 5
Fall	CFS-2 thru 7	10 ± 20	53 ± 32*	5 ± 8	14 ± 18	4 ± 11
Winter	CFS-2 thru 7	14 ± 15	9 ± 20	13 ± 25	12 ± 18	18 ± 24

*Significantly different from Winter; CFS-1: upstream from sites impacted by cattle; CFS-2 thru 7: farm sites potentially impacted by cattle.

Seasonal and Spatial Variability of Enterococci Communities in Manure and Water. Cluster analysis was performed on the relative % abundances of the five most common enterococci groups and the general enterococci category found in the stream water and manure samples. The purpose of the analysis was to determine if a community approach could produce useful information related to developing more reliable MST data analysis. The analysis produced 15 clusters of species that were identified as being present in the system three or more times through out the whole sampling period (Table 5).

Table 5: Composition and abundance (%) of *Enterococcus* species in clusters that appeared 3 or more times in water and manure samples collected at a Georgia cattle farm from September 2003 through January 2005.

	% Composition of each *Enterococcus* species				
Cluster Composition	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
E. casseliflavus	14	5	0	39	67
E. faecalis	43	0	32	21	0
E. faecium	0	0	5	3	0
E. flavescens	0	0	0	0	0
E. hirae	43	95	37	13	33
All other Enterococcus	0	0	26	24	0
	Cluster 6	Cluster 7	Cluster 8	Cluster 9	Cluster 11
E. casseliflavus	11	84	18	23	44
E. faecalis	3	0	31	61	0
E. faecium	82	4	0.3	0	0
E. flavescens	3	9	34	11	0
E. hirae	0.9	0	4	1	17
All other Enterococcus	0.9	3	11	3	38
	Cluster 12	Cluster 13	Cluster 16	Cluster 17	Cluster 18
E. casseliflavus	0	0	28	0	0
E. faecalis	0	100	0	0	77
E. faecium	0	0	7	0	23
E. flavescens	100	0	22	0	0
E. hirae	0	0	0	0	0
All other Enterococcus	0	0	43	100	0

Six enterococci clusters were found in high relative % occurrence for the three different sample sources, i.e., upstream-of-the-farm, at-the-farm sites and manure (Figure 6). Cluster 4 was only found at the upstream and farm locations where *E. casseliflavus* was usually more abundant. Clusters 8, 9, and 13 were not only present in manure, but were also frequently found in the upstream and farm samples; *E. faecalis* was overall the most abundant species in these three clusters. Clusters 6 and 7 were only found at-the-farm sites and manure

samples, and had a higher occurrence in the manure samples. *E. faecium* and *E. casseliflavus* were the most abundant species in these two clusters (Figure 6).

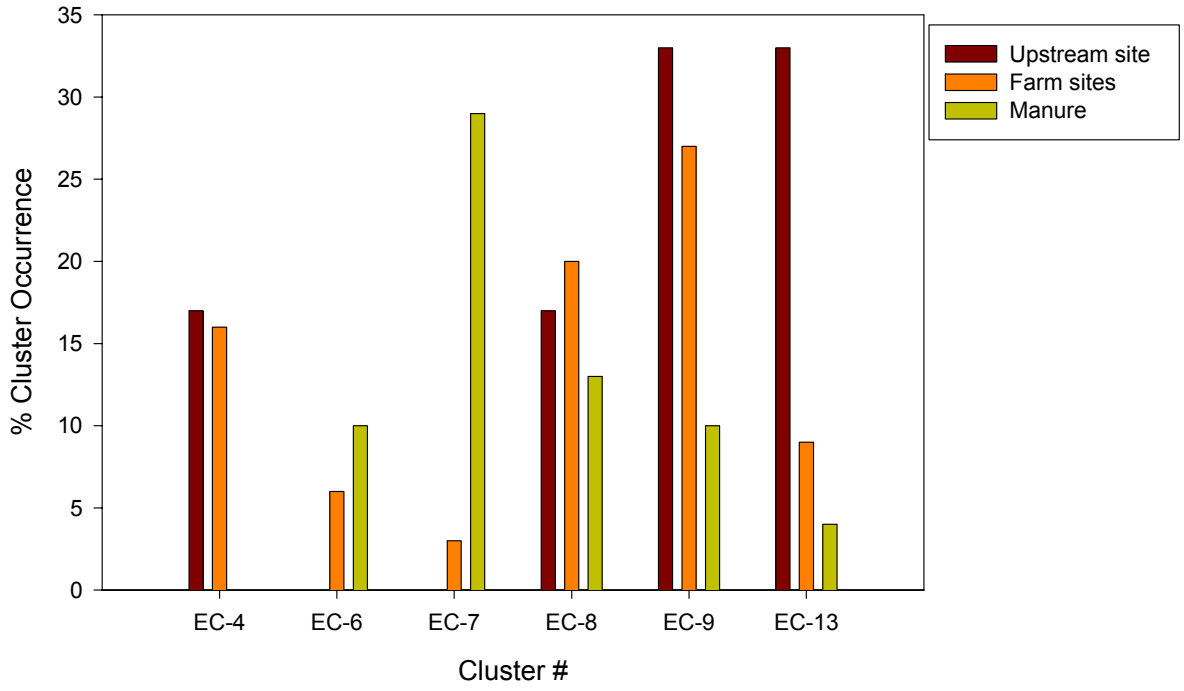

Figure 6: High % occurrence of *Enterococcus* clusters (EC) in samples collected from different sources at a cattle farm in Georgia.

Eight clusters were found only in manure and at-the-farm samples (Figure 7). However, these clusters were generally composed of species found at lower abundances (8% or less) in the sampled material. *E. casseliflavus* and *E. hirae* were predominant in clusters 1, 2, 3, 5, 11, and 16; in addition, clusters 1 and 3 also contained significant contributions of *E. faecalis* that also dominated cluster 18.

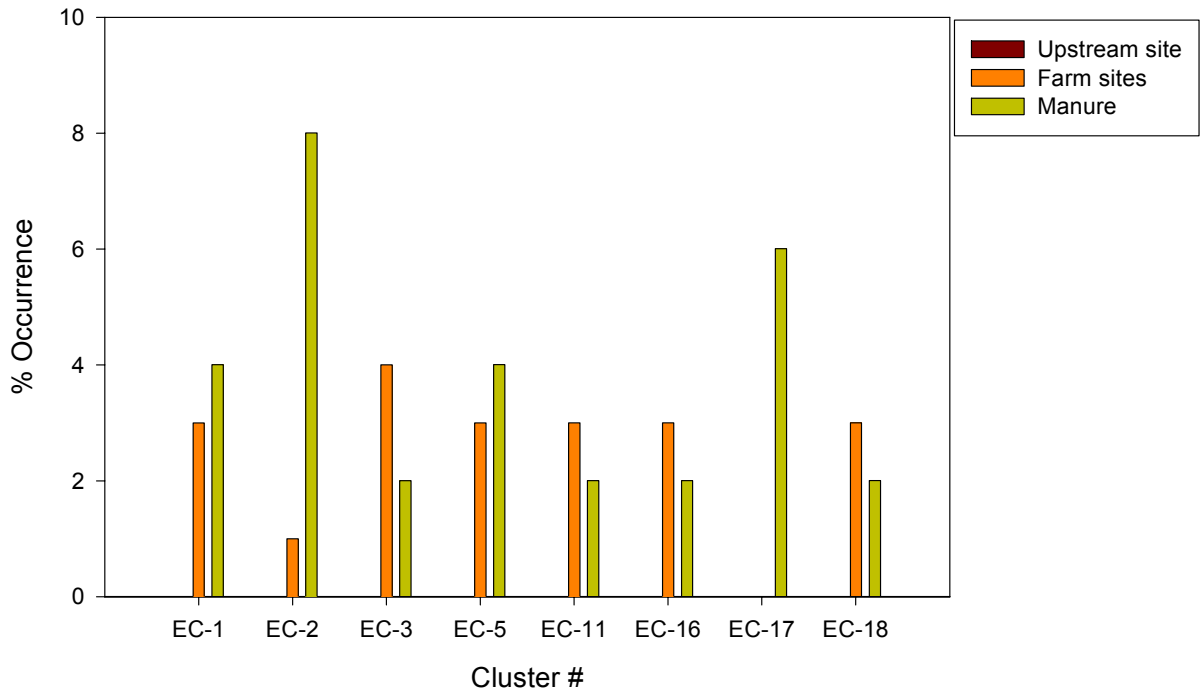

Figure 7: Low % occurrence of *Enterococcus* clusters in samples collected from different sources in a cattle farm.

These data suggest that the % distribution of specific species of enterococci in a community (cluster) might be an indication of the source environment. The results also indicate that same communities (clusters) found in manure are also found in stream water samples impacted by cattle (Figure 7) despit their low % abundance. However, it seems that such indicators could be highly seasonal. For instance, those clusters only found in manure and farm water samples were present during the cooler months (spring, fall, and winter), but were never observed during the summer (Figure 8). The most frequently observed clusters were found through out the four seasons, but seasonal differences can still be seen. For instance, EC-4 is clearly more frequently

observed during spring, while for EC-9 is clearly more abundant during fall (Figure 9).

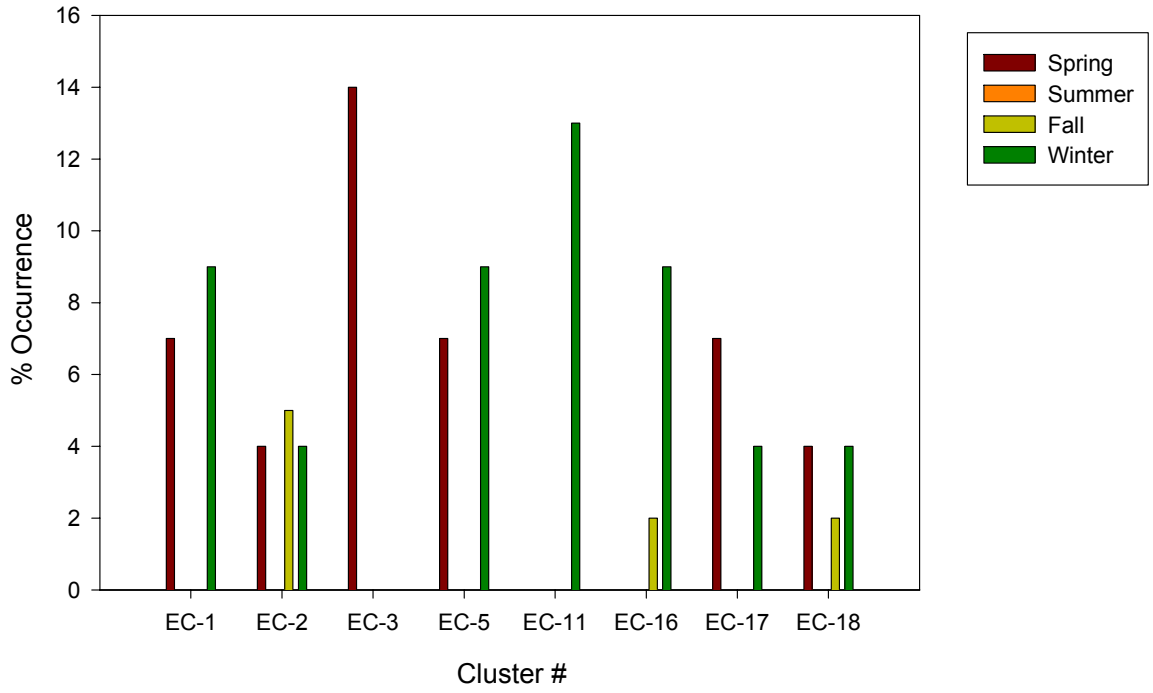

Figure 8: Low % occurrence of enterococci clusters during different seasons in samples collected at a cattle farm in Georgia.

The higher observed frequency for EC-9 during fall is basically due to the significantly higher concentration of *E. faecalis* in the water samples (Table 4). In the summer, *E. casseliflavus* and *E. flavescens* were highly abundant in clusters 7 and 8, respectively. (*Note: E. casseliflavus and flavescens have been re-classified as the same species under E. casseliflavus (Gilmore, 2002)*).

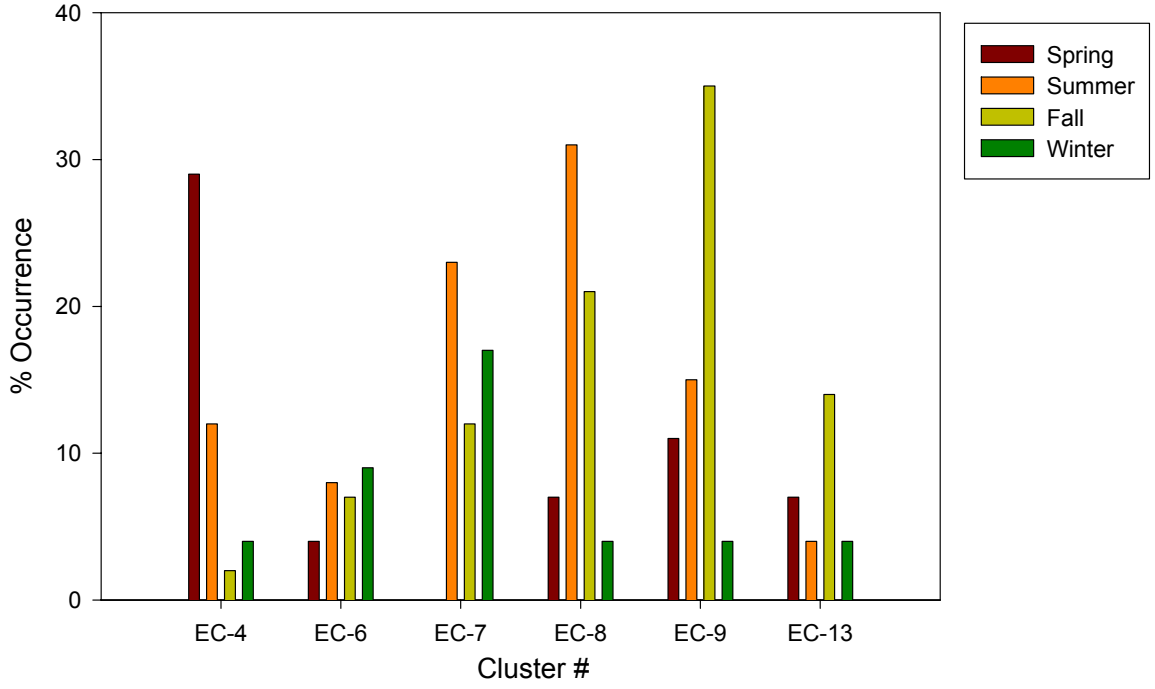

Figure 9: High % occurrence of enterococci clusters during different seasons in samples collected at a cattle farm in Georgia.

Figure 10 shows the distribution of clusters and % occurrence of each cluster per sampling site. The figure shows that only one cluster, although widely observed in the water samples, could not be found in the manure samples (EC-4). Likewise, EC-12 and EC-17 were only found in manure but not in the water. These clusters are composed of *E. flavescence* and enterococci that could not be speciated. Although a variety of clusters could be found at any given time at each water sampling station, the most clusters identified per site was 8, while 14 clusters were identified in the manure samples, which indicates a much larger diversity of enterococci communities in the cattle GI system. The upstream site (CFS-1) with only 4 clusters, had the least diversity found in the system. The

clusters present at CSF-1 are part of the background composition of the system because they are present at all the water sampling sites. In almost all the farm stream sites, the cluster diversity was higher, probably reflecting the effect that the manure added to the system.

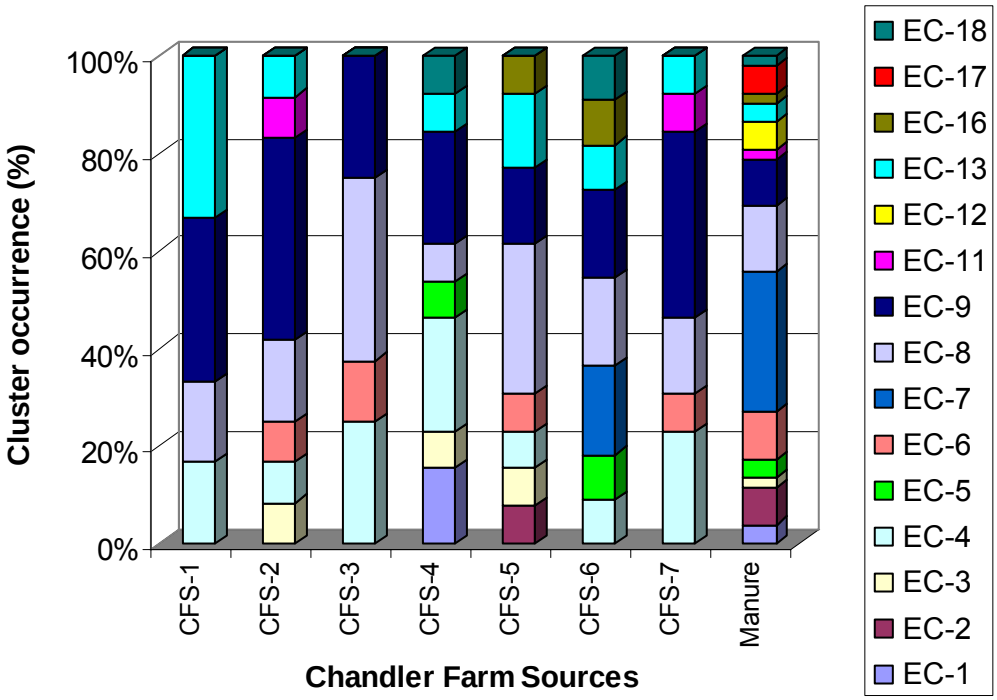

Figure 10: Cluster distribution and occurrence (%) per Chandler Farm sampling site and source.

Comparison of *Bacteroides* Markers and Enterococci Clusters. The *Bacteroides* markers (BM) were organized in five different clusters (Table 6) and this information compared to the presence of the enterococci clusters (EC) in the water samples (Figure 11). The human-BM was found twice concurrent with EC-8 and once with EC-9. These two clusters had high abundance of *E. faecalis* and *E. flavescens*. The cow-BM was found concurrent with 6 different ECs, but

again most frequently with EC-8 and EC-9. In addition, both BMs were found with EC-1 at least once. No spatial trend for either BM could be established, which means that the markers were found at various locations in the stream through out the year. Two possible conclusions can be drawn from these results. The fact that the human-BM was found in various locations in the farm stream water suggested that some of the *E. faecalis* and *E. flavescence* in the water may not be coming only from cattle or wildlife, but also from human contamination. The sources for this contamination could be leaky septic systems given the rural aspect of the location where the samples were obtained. Alternatively, these results could suggest that the human-BM was amplifying *Bacteroides* DNA from sources other than human. This latter hypothesis also applies to one of the cow-BMs that was amplified at CFS-1, the site upstream from obvious areas of cattle contamination. This site could be affected by run-off coming from various chicken houses located in fairly close proximity to the stream headwaters (see Figure 2). These chicken houses could also be responsible for the higher-than-expected enterococci diversity in the stream water.

Table 6: Composition of *Bacteroides* clusters identified in stream water collected at a cattle farm in Georgia

	Presence of marker[1] in cluster		
Bacteroides Cluster #	General Marker	Cow Marker[2]	Human Marker[2]
1	Yes	No	No
2	Yes	No	Yes
3	Yes	Yes	No
4	Yes	Yes	Yes
5	No	No	No

[1] Reference for all *Bacteroides* markers: Bernhard and Field, 2000.
[2] The two human markers and two cow markers were combined to develop clusters 2 and 3, respectively

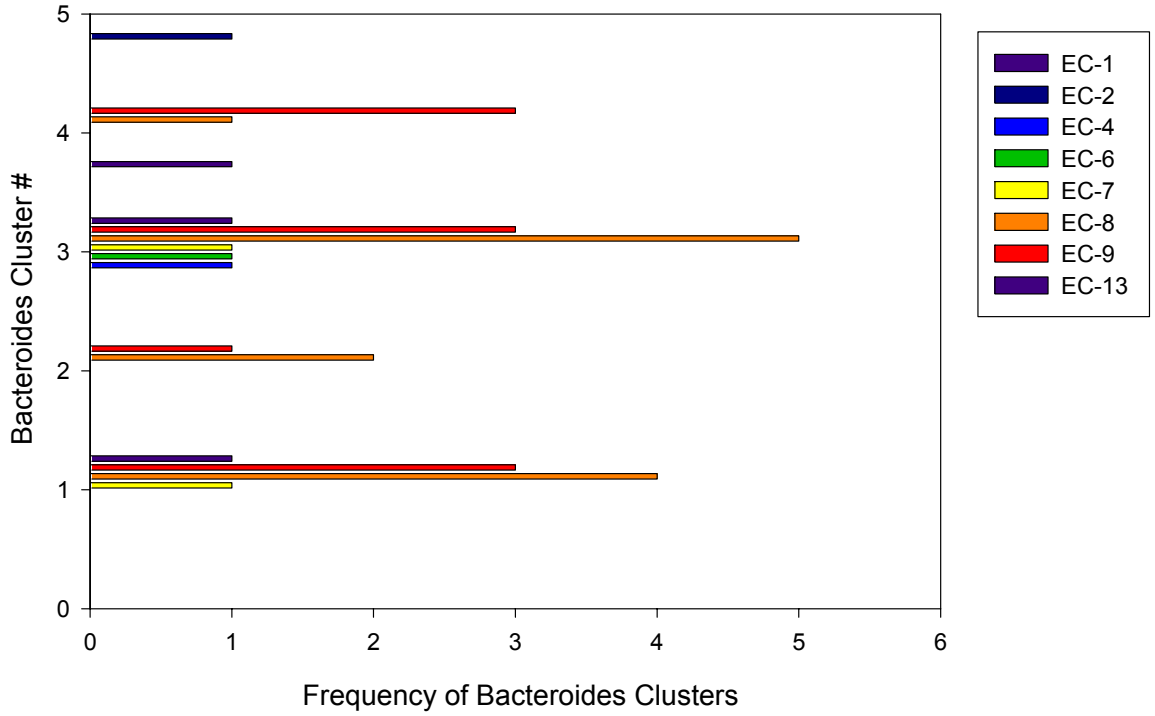

Figure 11: Relationship of *Bacteroides* and enterococci clusters in stream water samples collected at a cattle farm in Georgia.

Conclusions and Final Considerations

The general conclusions for this study follow:

- From a total of 11 *Enterococcus* species that were identified at Chandler farm, 2 were only found in cattle manure, but were not recovered in stream water. This makes such species unreliable markers of cattle fecal contamination in surface waters since they do not survive in this environment.

- The relative abundance of individual *Enterococcus* species isolated from cattle manure that were also observed in the stream samples exhibited a high degree of seasonal variability. This finding suggests that when tracing back cattle contamination, season should be an important consideration to include in the criteria to select the species that can be used as tracer. However, the high degree of seasonal variability in some of the most common species makes it very difficult to establish significant differences between seasons and /or the sampled sources.

- The 5 most common enterococci species identified were found in the water samples at-the-farm and upstream-the- farm locations, suggesting that these species are widely spread in the environment. Wildlife, an adjacent-to-the-farm chicken house, and a few scattered single-family houses could be contributing these same species in high numbers, therefore creating high background concentrations.

- Cluster analysis seems to be a good approach to identify species groups or enterococci communities that are specific to a location or source, and suggests that a community fingerprint rather than an individual species could be an alternative approach to trace back stream fecal contamination to its source.

- Results with the *Bacteroides* markers generally agreed with the enterococci data in that water sampled from stream locations CFS-2 thru 4 was highly impacted by cattle contamination, while locations CFS-5 thru 7 had occasional hits apparently affected by the season of the sampling event. However, the cow marker was also detected at location CFS-1 that was not under obvious cattle influence. The human B*acteroides* marker was also detected occasionally throughout all stream locations, except for CFS-1, indicating either human fecal contamination in parts of the stream or non-specific amplification of the human- and cow-bacteroides markers due to other sources, such as poultry manure which is frequently used to fertilize cattle pasture sites.

- The two methodologies applied in this study differ greatly in terms of cost effectiveness and turn-around time of results. Building an enterococci library is a time-consuming, expensive approach that has the potential to provide a great deal of information when the proper statistical analytical approach (in this case it was cluster analysis) is used to interpret the results. Time availability (when are results expected or needed) and funding support (large quantities of consumable laboratory supplies are needed) are two important considerations to keep in mind when a library-dependent method for microbial source tracking is planned. Application of a library-independent approach, such as the *Bacteroides* markers allows for a much faster and possibly less expensive results. However, the need still exists for highly specific, reliable markers that will allow one to separate specific sources and not only human vs. non human contamination. In the case of *Bacteroides*, there remains a lack of thorough temporal, spatial and specificity analyses of the few genetic markers available so far.

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