Upper Fish River Bacterial Source Tracking Project
October 2008 - March 2011

Final Report for the Mobile Bay National Estuary Program

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Final Report

Introduction

Fish River in the Weeks Bay watershed, Baldwin County, Alabama is included on the Clean Water Act §303(d) list for pathogen contamination. The upper reaches of the Fish River (HUC 031602050201) watershed were identified by the Mobile Bay National Estuary Program (MBNEP) as a priority area. The land use is mixed and includes urban development, agriculture, pasture and forests (Figure 1). Even though a mixture of uses is present, the dominate use in the upper Fish River remains agriculture (NRCS). Also, Fish River is one of the two main tributaries to Weeks Bay, a designated "Outstanding National Resource Water." Pathogen contamination in the river and the potential human health threat associated with these bacteria are identified as an environmental problems in the Weeks Bay Watershed Management Plan. Typically, potential sources of pathogen contamination have not been identified, only simply recognized; *e.g.* there are cows in the stream, cattle are known sources of fecal bacteria; therefore if the cows are removed, the pathogen source will be removed. Efforts in the past have done just that; cattle fenced out, provided an alternative watering source and provided a hard-bottom crossing. Yet in many cases, pathogen counts have remained high. As for Fish River, this is the reality. The Weeks Bay Foundation (Foundation) has funded bacterial monitoring in cooperation with the volunteer water monitoring group, Weeks Bay Water Watch. In addition, the Foundation has partnered and funded joint pathogen monitoring efforts with Weeks Bay Reserve, a partnership between the Alabama Department of Conservation and Natural Resources, State Lands Division (ADCNR/SLD) and the National Estuarine Research Reserve System of the National Oceanic and Atmospheric Administration.

Current fecal coliform monitoring includes locations spanning much of the accessible reaches of Fish River and several tributaries. Counts of bacteria in the upper Fish River remain periodically high and exceed the limits of its water use classifications, Swimming and Fish and Wildlife, as established by the Alabama Department of Environmental Management (ADEM). High counts typically follow rain events. Even though pathogens in Fish River are actively enumerated, there have been no detailed studies examining the source(s) of contamination at the cellular level. As stated, high counts typically occur during high water events. A clear need existed to better identify sources so that better management programs may be developed to address the water quality problem. Potential sources like pasture grazing remain, but with continuing development occurring in the upper Fish River watershed additional sources like failing septic systems, sewer line malfunction, discharge from wastewater treatment plants and urban stormwater runoff become more prevalent. Knowing the sources and how they are (or are not) affected by rainfall events will aid in making better management decisions and ultimately reduce pathogen pollution.

In this study, two well-tested source tracking methods were used to examine the origins of bacteria in the upper reaches of Fish River. The study area started just upstream of

the confluence with Polecat Creek near Silverhill, AL northward to the source. The comparison of growth patterns of pathogens in the presence of a variety of antibiotics has been used in watersheds in Alabama as a source identification tool. Antibiotic resistance testing was used in this study as one technique for source identification. *E. coli* was used as the indicator bacteria for this investigation. Cells from known sources (human, cow and horse) were grown in the presence of several antibiotics and their growth patterns were statistically analyzed to discriminate between the sources. The growth patterns of *E. coli* collected from Fish River water were compared to the patterns of the known sources. Discriminant analysis, a statistical method, was used to separate growth patterns into classifications. The cells collected in Fish River were classified into human, cow or horse patterns. The next technique involved the use of unique DNA sequences to identify the source of bacteria that are strong indicators of the presence of pathogenic strains. The DNA-based source detection methods selected for this study are reliable and available at several commercial laboratories. These methods rely on amplification or increasing the number of DNA sequences (markers) unique to specific warm-blooded animals including humans.

This report includes historical information about *E. coli* counts at the downstream-most Fish River sampling site in the study - Woodhaven Dairy Road. A detailed examination of the increases in *E. coli* counts in response to rainfall events is also included. Throughout the study, volunteers faithfully documented rainfall amounts and reported qualifying storm events for water collection. Building in the preparatory data and rainfall monitoring, two bacterial source tracking methods were used to identify sources of bacterial contamination. As part of the project, a student in the International Baccalaureate program at Fairhope High School was able to complete a significant part of his study, the Extended Essay, while working on this project. In addition, undergraduate students at the University of West Alabama were able to advance their knowledge of laboratory work and microbiology while conducting antibiotic resistance analysis. Finally, this study could be reproduced in other watersheds including those in middle and lower Fish River to identify contamination source. Management measures can be enhanced by the results of this study to reduce or eliminate bacterial pollution in the Fish River.

Materials and Methods

Rainfall Monitoring

Volunteers were recruited to monitor rainfall and be sentinels for rain events that would trigger river water sampling. Monitors were selected for their locations along the reach of Fish River within the upper portion of the watershed. All lived in close proximity to the sampling sites (Figure 2). Oregon Scientific™ Model RGR682 (Cannon Beach, OR) electronic rain gauges were installed at each location. The electronic gauge consists of an outdoor self-tipping bucket with built in sending unit and an indoor receiver. The receiver tracked both daily and cumulative rainfall. A nine-day memory was maintained by the unit. Daily rainfall was reset to zero at midnight on each evening, so daily rainfall was collected from midnight to midnight as a 24-hour cycle.

The total rainfall was collected over time providing a long-term assessment of rain amounts over the monitoring period. Volunteers were provided monthly rainfall data sheets and instructions for recognizing gauge problems. The units were purposely selected for their ease of operation. Volunteers were instructed to diagnose problems and immediately contact project manager for maintenance or repair. Once installed, the outside unit needed to remain level to function correctly, so all troubleshooting was left to the project manager.

Volunteers were instructed to check rainfall monitor and record data at 1900 hours (or 7:00pm) each day or as close to that time as is practicle. When a DAILY total of 0.1 inches or greater was recorded, volunteers were instructed to contact the project manager by phone or email to report qualifying rainfall event. Summary of rainfall data is depicted in Figure 3.

E. coli Collection

E. coli were collected using Coliscan Easygel (Micrology Laboratories, Goshen, IN). Fish River water samples were taken from a flowing portion of the waterway in sterile plastic bottles. Details of Easygel method are described in Appendix A. Locations of water sampling sites are identified on Figure 4. Water samples were collected during both high water events resulting from rainfall and baseflow conditions. *E. coli* from known sources (human, bovine and equine) were collected at several sites inside and outside the Fish River watershed (Figure 5). Human *E coli* were collected from centralized wastewater treatment facilities in Loxley, Foley, Fairhope and Daphne. Treatment plant influent was collected in sterile bottles, diluted using commercially available bottled water and planted to achieve 12-25 *E. coli* colonies per plate. The exact number of colonies per 100 ml was not enumerated. Fecal material from cows (bovines) and horses (equines) was collected at several sites both inside and outside the watershed of Fish River (Figure 5). Fecal material was collected in plastic bags. The solid material was diluted using commercially available bottled water and plated to achieve a 12-25 *E. coli* colonies per plate. The exact number of colonies per 100 ml was not enumerated. Sample plates were incubated at 36-37°C for 24-48 hrs. Description of collection is summarized in the extended essay written a Fairhope High School student as part of the International Baccalaureate course requirements (Appendix B). Plates were shipped unchilled and overnight to University of West Alabama for antibiotic resistance analysis.

Antibiotic Resistance Analysis

Antibiotic resistance analysis was used to identify the likely source(s) of the bacteria. Antibiotic resistance patterns were performed according to Burnes B.S. (2003) **Antibiotic Resistance Analysis of Fecal Coliforms to Determine Fecal Pollution Sources in a Mixed-Use Watershed,** Environmental Monitoring and Assessment, Volume 85, Number 1, pp. 87-98(12) and Wiggins, B.A. et.al. (1999) **Use of Antibiotic Resistance Analysis to Identify Nonpoint Sources of Fecal Pollution**, Applied and Environmental Microbiology 65:3483-3486. Discriminant analysis was performed

according to Burnes B.S. (2003) **Antibiotic Resistance Analysis of Fecal Coliforms to Determine Fecal Pollution Sources in a Mixed-Use Watershed,** Environmental Monitoring and Assessment, Volume 85, Number 1, pp. 87-98(12);Wiggins, B.A. et.al.(1999) **Use of Antibiotic Resistance Analysis to Identify Nonpoint Sources of Fecal Pollution**, Applied and Environmental Microbiology 65:3483-3486 and Wiggins, B.A., (1996), **Discriminant Analysis of Antibiotic Resistance Patterns in Fecal Streptococci, a Method to Differentiate Human and Animal Sources of Fecal Pollution in Natural Waters**, Applied and Environmental Microbiology 62:3997-4002.

Samples were also collected from probable sources of pathogen contamination in the Fish River watershed. These samples were from a municipal waste water treatment plant (for human *E. coli*), pasture-kept cattle (for bovine *E. coli*), and pasture-kept horses (for equine *E. coli*). In order to isolate *E. coli,* each sample was serially-diluted and inoculated into Coliscan Easygel plates according per the manufacturer instructions. A total of 860 *E. coli* isolates were collected throughout the study period, including 651 *E. coli* isolates from river water samples and 209 *E. coli* isolates from known sources. A reference strain, *E. coli* no. 11775 (American Type Culture Collection, Rockville, MD), was included as a positive control.

All *E. coli* were purified and assayed for growth in the presence of 13 commonly-used antibiotics (Table 1). Each isolate was given a unique sample number, sample date and *E. coli* strain designation. Resistance was scored in growth assays. These results were then compared to identify patterns that linked *E. coli* from the Fish River to *E. coli* that were human, bovine, or equine in origin. The method used to identify the patterns is discriminant function analysis, a type of multivariate statistical analysis designed to recognize patterns and classify unknown cases into known groups. The inclusion of three distinct source groups in this study allowed the generation of two discriminant functions. The equations for the discriminant functions are:

DF1 = -3.249 +0.136Amp +0.074Cip +0.094Ery +0.207Naa +0.458Str
DF2 = -5.249 +0.133Amp +0.242Cip +0.179Ery +0.417Naa +0.355Str

The two discriminant functions were applied to the growth assay results of every *E. coli* isolate, resulting in two discriminant function scores for every isolate. The origin, discriminant function scores, classification, probability, and distances from the group center of all *E. coli* isolates are listed in Appendix C.

DNA-based Source Identification Testing

Samples for DNA-based source identification testing were collected in sterile plastic bottles and chilled on ice. The laboratory tested each sample for the indicator bacteria prior to DNA amplification, so enumeration of *E. coli* in the river sample was not conducted on each sample. Bottles containing river water for testing were placed in plastic zipper-seal bag and packed on ice in a rigid cooler. Coolers were shipped overnight to Source Molecular Corporation for appropriate testing. Details of testing methods are contained in Appendix D.

Results

Examination of the *E. coli* counts in Fish River in response to rainfall events.

Prior to the testing accompanying this project, enumeration of *E. coli* was carried out by a volunteer participating in the Alabama Water Watch program in the watershed of Weeks Bay. The site on Fish River located north of the confluence with Polecat Creek on Woodhaven Dairy Rd. was included as a source tracking site because of the long data history. In addition, the volunteer has collected precipitation at the sampling location for several months prior to initiation of this project. In addition, precipitation and river discharge data is available from the U. S. Geological Survey gage station on Fish River at Alabama Highway 104. The river gage is located about four miles north of the Woodhaven Dairy Rd. Historically, *E. coli* counts increase in response to rainfall events (Table 2). Typical *E. coli* counts at the site range from 20-80 colonies per 100 ml. On 1/27/08 and with a total amount of 2.53 in. accumulating in the previous three day, a count of 1,833 colonies per 100 ml was recorded. A similar result was recorded on 2/15/09. On other dates with increased rain amounts on or leading up to the sample date like 7/14/07, 5/18/08, 6/15/08 and 11/30/08, bacteria counts were elevated, but not above 1,000 colonies per 100ml. Bacteria counts seemed to show little or no increase resulted from increased rainfall on 7/14/07 and 9/21/08.

Since project initiation, *E. coli* counts in Fish River at the Woodhaven Dairy Rd. location were enumerated in response to selected rain events (Table 3). *E. coli* sampling events were triggered by a 0.1 in rainfall event provided that no rain was recorded in the previous 72 hr. Water samples were taken for *E. coli* enumeration twice daily for five events in 2008. In the first sampling in response to only 0.15 in. of precipitation (10/18/08), *E. coli* counts remained at typical concentrations. One week later in response to a 2.5 in rain event (10/24/08), *E. coli* counts ramped up quickly and diminished back to typical levels within about two days. A modest increase in *E. coli* counts occurred in November sampling events when 0.69 in (11/8/08) and 0.32 in (11/25/08) were recorded. Four days following the 11/25/08 testing, an additional 1.8 in rain fell at the sample site resulting in an increase in counts (11/29-30/08). All the sampling events in 2009 revealed similar results (Table 2). *E. coli* counts increased rapidly to 1600-2200 colonies per 100ml. Counts decreased rapidly also. In April 2009, counts spiked to 2722 colonies per 100 ml after a rainfall event of 0.78 in. Rainfall data reveal the need to sample the river early in the rainfall event to maximize the chance of capturing the increased counts. Counts in the river increase rapidly and decrease nearly as rapidly.

Rainfall Monitoring

Volunteers responded to the search for sentinels to monitor rainfall events and contact the Project Manager to collect water samples. The more ambitious plan to train volunteers to collect water samples was discontinued based upon the safety issue of

sampling during times of high water. Six monitoring stations were established at locations near the water sampling stations (Figures 2 and 4). Both daily and cumulative rainfall data was collected each day. Monitors at locations near the US 90, CR 54 and Woodhaven Dairy Rd submitted data with most regular frequency and for the longest duration of time (Figure 3). Also, these volunteer monitors consistently reported qualifying storm events. In combination with gage height and discharge data available from the USGS gauge station on Fish River (USGS 02378500 FISH RIVER NEAR SILVER HILL AL), the volunteers collected data, and their willingness to report rainfall events facilitated water sample collection.

Sampling *E. coli* for Antibiotic Resistance Analysis

E. coli was enumerated at both low water (dry) and high water (rain) conditions at six locations along the upper reaches of Fish River (Figure 4). High water sampling was conducted in response to selected rainfall events of 0.1 in or greater. Sampling was conducted by the project manager. Only five *E. coli* colonies were detected in the January 2009 low water sampling (Table 6). Despite the low numbers, plates were shipped overnight to the University of West Alabama for antibiotic resistance analysis. The winter high water sampling event was performed February 2009, accompanying an over two inch rainfall event. Water samples were plated using three dilutions (Table 3). *E. coli* counts were elevated at each of the sites compared to the winter dry sampling. The fewest cells were enumerated at Fish River at Interstate 10, 199 colonies per 100ml. The highest concentration of *E. coli* was enumerated at Fish River at CR54, 1,555 colonies per 100ml. Again, plates were shipped overnight to the University of West Alabama for testing.

With less than 0.2 in rain recorded over two weeks by volunteers and USGS Gage Station on Fish River, the spring low water sampling was conducted in April 2009 (Table 4). Counts were low at each of the six Fish River sites. Five replicates were plated for each Fish River site. The highest concentration of *E. coli* at any site was 30 colonies per 100ml. The spring high water sampling event was completed in May 2009. Over three inches of rain was recorded by volunteer monitors. As in previous high water events, *E. coli* counts were high (Table 5). The highest counts were recorded at the CR48 site. Lowest counts were recorded on Fish River at Interstate 10. The rain event in May 2009 was the first significant amount of rain since mid-April. The build up of cells on the landscape without a flushing rain event likely contributed to the high counts. The remaining counts for *E. coli* submitted for antibiotic resistance testing are listed in Table 6. As in all other sampling events, plates containing *E. coli* colonies were shipped to the University of West Alabama for testing.

Sampling for known sources of *E. coli* was conducted at several sites within and outside the upper Fish River watershed (Figure 5). The antibiotic resistance growth patterns of the known sources were compared to the unknowns collected from Fish River. Human cells were isolated from influent water entering four local wastewater treatment plants: Baldwin County Sewer Service Plantation Hills, City of Loxley, City of Fairhope and Riviera Utilities, Foley. Influent water was selected as a source of *E.*

coli because untreated wastewater arriving at each of the plant is almost exclusively from residential sources implying human sources. Cells for bovine and equine sources were collected from manure samples. In the case of all known *E. coli* sources, source material (treatment plant influent or manure) was diluted to achieve coverage on the plates of 15-25 colonies. Two rounds of sampling for known *E. coli* sources were conducted: May 2009 and August 2010. Counts for each sample were not enumerated. For final antibiotic resistance growth testing and discriminant function analysis, sample size was about 90 isolates for bovine, 65 for equine and 52 for humans.

Gibbs Pearson, an International Baccalaureate student at Fairhope High School, assisted with the summer 2010 sampling of *E. coli* of known sources. As part of his International Baccalaureate study, Gibbs was required to conduct a research project and compose an extended essay summarizing the experience. The student was involved in both sample collection and isolation of *E. coli* samples. Gibbs' Extended Essay is contained in Appendix B.

Antibiotic Resistance Analysis

The two discriminant functions were applied to the growth assay results of every *E. coli* isolate, resulting in two discriminant function scores for every isolate. The origin, discriminant function scores, classification, probability, and distances from the group center of all *E. coli* isolates are listed in Appendix C.

The discriminant function scores of the reference *E. coli* isolates (human, bovine, and equine) were plotted with the DF1 score on the x-axis and the DF2 score on the y-axis (Figure 6). A territory map was delineated along the discriminant function minima at the lines defining each group. The accuracy of Figure 6 is assessed by calculating the percent of the known source *E. coli* isolates which are correctly classified into their groups of origin. The isolates in this study have an average correct classification rate of 61.9%. These results are significant, in that any classification rate above 33% is considered better than random. There is significant overlap in the discriminant scores of bovine and equine *E. coli*, which reduces the accuracy of differentiating between the two groups. The relative similarity of non-human discriminant function scores is consistent with previous studies and has been the basis for structuring other study comparisons as human versus non-human. The classification rates for each reference group and both sampling dates are shaded in Table 7.

Once the territory map was delineated, the *E. coli* collected from the Fish River were plotted in an identical manner to the known-source *E. coli* (Figure 7). The distribution of the probable sources of *E. coli* is weighted by the number of E. coli/ml determined from each sample. The total distribution of *E. coli* found in the Upper Fish River is:

16.4% of the *E. coli* were of human origin,
52.8% of the *E. coli* were of bovine origin, and
30.9% of the *E. coli* were of equine origin.

Individual discriminant score plots of all samples are available but not included in this report due to the 87-page length of the figure. The *E. coli* classifications from all

samples from the Fish River are listed in Table 8 and separated by sampling event in Figure 8. Variability in the source of *E. coli* is evident. No one source is consistently more abundant at any particular site. The low water samples taken in March 2009 contain a mix of source classification except for water taken at the CR 64 site. The CR 64 site contain no human derived cells. At the January 2009 high water sampling event, the human derived cells were detected in abundance at CR 64. Bovine and equine derived cells are abundant at all sites from January 2011, yet in the January 2009 sampling event, which showed similar overall *E. coli* counts to the 2011 event, there are sites with high numbers of human derived cells. Again, no clear pattern of dominance by one source or another is evident. When the results of high overall cell counts, like in the April 2009, May 2009 and Nov 2010 (1st Sampling) events, are examined, there are sites, I-10, CR64, CR48 and US90 with high bovine derived cells. The pattern does not remain consistent.

DNA-based Source Identification Results

To add further weight to determination of sources for *E. coli* in Fish River, DNA-based source identification testing was conducted on water collected at Woodhaven Dairy Rd, CR54 and US90. Human and bovine markers from two classes of indicator bacteria, *Enterococcus* and *Bacteroidetes*, were used to probe bacteria found in water from Fish River. Equine markers for *Bacteroidetes* were available to probe water samples. The number of sample sites was reduced to three, due to the cost of testing. As with water samples collected for antibiotic resistance testing, river conditions of both baseflow (dry weather) and high (rain conditions) water were sampled. Equine markers were used to probe on water samples collected under rainfall conditions. The DNA-based tests were more sensitive and reflected accurately the source of indicator bacteria even though few cells existed. Also, prior to each analysis, water samples were tested for a minimum number of cells for each bacteria class, *Enterococcus* and *Bacteroidetes*. The results of the tests were detected or not detected indicating only the presence or absence and not relative abundance.

Human markers for neither indicator bacteria were detected under low water conditions in the single sampling event in April 2010 (Table 9). Human *Enterococcus* markers were detected under the two high water events sampled but on at the same location. In May 2010, the marker was detected at Woodhaven Dairy Rd, but in January 2011, it was detected at CR54. Human *Bacteroidetes* markers were detected in the high water samples collected at each of the three sites in January 2011 but not in the high water sample collected in May 2010 or the baseflow sample taken in April 2010. Bovine markers for *Enterococcus* were detected at two of the three sites tested in April 2010: CR54 and Woodhaven Dairy Rd. Bovine *Bacteroidetes* markers were not detected at any of the three sites under either baseflow or high water conditions (Table 10). No bovine *Bacteroidetes* markers were identified in any sample collected. Under the high water conditions, no markers from either indicator bacteria were detected at any of the three sites. *Enterococcus* markers were not available for any equine samples so only *Bacteroidetes* markers were used to probe Fish River samples. Only high water

conditions were tested. Markers were detected at all three locations in February 2011 and at CR54 in March 2011 (Table 11).

Conclusions

The watershed surrounding the upper reaches of Fish River contains a variety of uses, all of which have the potential to contribute *E. coli* and other pathogens to the river. Currently two wastewater treatment facilities discharge directly into the river. Both plants have remained in compliance with their discharge limits, yet centralized treatment works like these and the pipes and lift stations that are part of the system have the potential to fail or leak. Residential areas can contribute harmful bacteria through failing septic tanks and pet waste. Pasture grazing by cattle and the manure they leave behind can contribute pathogens to Fish River and its tributaries. Even though various wildlife populations have not been enumerated in the upper Fish River watershed, there is a high likelihood that the forested area in the landscape should support large populations of various species. In this study, the sources of pathogens in the Fish River were examined. *E. coli* of unknown sources isolated from the waters of the upper reaches of Fish River were compared to three from the most likely known sources: human, cattle and horses.

It has been established through historical testing and in work conducted as part of this study that rainfall has a dramatic effect on the numbers of *E. coli* in the river. The concentrations of bacteria jump from nearly zero to several thousand per ml? in response to rainfall events. Examination of the numbers of cells in response to different rain events reveals the need to sample the river early in the rainfall event to maximize the chance of capturing the increased counts. Counts in the river increase rapidly and decrease nearly as rapidly, suggesting that the source of the *E. coli* is surface runoff. If sources were predominately in the groundwater, counts would not react as quickly to rain events. Understanding that the sources likely contributed through surface runoff is important. This fact will help educate future planning and management decisions.

Two methods were used in this study to identify sources of the *E. coli*. Both methods have been used successfully in other watersheds. The first method was multiple antibiotic resistance analysis. This method exposes *E. coli* to a variety of antibiotics, and subsequent growth patterns are examined. Growth patterns of known sources of *E. coli* were compared to growth patterns of cells of unknown sources collected from Fish River. The comparison is made using a statistical application called discriminant analysis. Because the high number of cell examined in this study and the separation afforded by analysis of the growth patterns of *E. coli* from know sources, confidence in the statistical results was high. Results showed that 16.4% of the *E. coli* cells were of human origin, 52.8% of the *E. coli* cells were of bovine origin, and 30.9% of the *E. coli* cells were of equine origin. Conventional wisdom would support the results for human and bovine. The upper Fish River area has been developing over the last two decades. Onsite sewage treatment persists. Increases in domestic sources of pathogens are likely. As stated earlier, two centralized domestic wastewater treatment plants are located in the upper watershed. Their buried lines enervate the area and their treated

water is discharged into the upper Fish River. Even though, no violations of discharge limits for either facility have been recorded recently, the plants remain potential pathogen sources. Cattle have been a mainstay of agriculture in the upper watershed. Grazing cattle are a prominent feature of the landscape. According to the Alabama Agricultural Statistics Service, Baldwin County produced about 23,500 head of cattle in 2011, yet those numbers have been declining. In 1995, total Baldwin County cattle production was 42,500 animals. The watershed surrounding the upper Fish River remains agricultural. Combining the residential development with the remaining large-scale presence of cattle grazing activities support the results produced by the antibiotic resistance testing: nearly 70% of the *E. coli* examined were from human or bovine sources. The more unexpected result of the testing was the almost 31% of the *E. coli* derived from equine sources. Horses have not been considered a significant source in the past, yet any future pathogen management plans must consider horses as a significant source. Overall, discriminant analysis does not show any consistent pattern based on season, month or rain event. The results support the idea of a watershed of mixed use contributing *E. coli* from a variety of sources.

The DNA-based testing carried out to confirm results of the antibiotic resistance work verify the presence of human, cattle and equine sources. Two indicator bacteria markers for human and bovine sources were used. Only one indicator was currently available for detection of bacteria from horses. The tests are presence-absence tests, yet both are sensitive to low concentrations of cells present in the water. Horse (equine) markers were detected in water from at least one location sampled in response to a rain event. The CR54 site showed the presence of horse markers at both testing events. Human markers were detected in only the high water samples taken. Even though the methods used to search for bovine markers are sensitive, no *E. coli* markers from bovine sources were detected in the high water samples. Only bovine *Enterococcus* was detected at all and only at the low water sampling event. This result is at odd with results seen in the antibiotic resistance testing, yet the presence of cattle markers at low water does still support the notion that cattle remain a significant source of bacteria. The cost of the DNA-based testing did force a reduction in the number of sites tested and the number of sampling events. The conclusion reached as a result of the two source tracking methods is all the sources examined remain serious potential sources in the upper watershed of Fish River. Human and equine sources seem a more significant threat following rainfall events. As in the past, management considerations for all three sources will have to be made in order to reduce pathogen number in Fish River.

Contributions from wildlife remain undertermined. Development of a classification system for the wide variety of animals that could contribute fecal material to the upper Fish River area was time- and cost-prohibitive. Statistical analysis used in this study could have classified *E. coli* cells into the three known categories examined. Examination of the classification data does indicate that some isolates weakly classified into each category. These cells could be from unknown origin but due to the classification statistics landed in a known category. Further collection of fecal material for wildlife and additional antibiotic resistance analysis could distinguish those *E. coli* isolates classifying more strongly into a separate wildlife category.

Results of this study can be used to educate future management decisions that will be made to address pathogen issues in the upper Fish River. A TMDL study by ADEM is not scheduled until 2013 (2010 303(d) List). The information could also be used to aid the Gulf of Mexico Initiative being conducted by NRCS in the upper Fish River watershed. The watershed was identified as a priority. Reduction of pathogens is one of the outcomes NRCS hopes to achieve. Identification of cattle or horse operations that could quality for USDA or NRCS cost-share programs would be an initial step in addressing pathogens inputs. Working with livestock owners to implement practices that reduce grazing activities close to intermittent or perennial streams or watering in the creeks around Fish River will contribute to reduction of pathogen inputs. There is a history in the Fish River watershed of practices intended to reduce pathogens and resulting in the removal of Caney Creek from the impaired waters list. A cattle owner on the creek worked with the Weeks Bay Watershed Project, NRCS and ADEM to fence cattle from the water and install a hard-bottom cattle crossing. Pathogen counts were reduced and the creek was removed from the 303(d) list. The U. S. Environmental Protection Agency recognized the effort in December 2007 (EPA 841-F-07-001EE). The cooperation afforded by the wastewater treatment plant operations could continue with the identification of training needs and other practices that could prevent or reduce upsets or overflows that result in sewage spills. Grease education programs have been successful in Daphne, AL and could be replicated. Engaging the Alabama Public Health Service and ADEM to identify areas where septic tanks exist could yield reduction in pathogen inputs. The Clean Water Partnership and MBNEP have cooperated on Juniper Creek and Eight Mile Creek in Mobile to address septic tank issues. Additional funding will be needed to support further pathogen reduction efforts.

News Coverage of Upper Fish River Source Tracking Project

The Upper Fish River Source Tracking Project received media attention in February 2009 with two newspaper articles. The first appeared on February 8, 2009 in the Baldwin County section of the Press-Register (Mobile, AL) written by staff reporter Ryan Dezember. The second appeared on February 16, 2009 in the Baldwin County regional paper, The Fairhope Courier, written by Curt Chapman. Both articles were informative and captured complex subject matter in a way that a general audience could understand. The Project Coordinator received many contacts as a result of the articles. A copy of the Fairhope Courier article was included in the letter sent to Fish River residents to recruit potential rainfall monitors. Copies of the articles are included in the Appendix F.

Scientific Presentations

Presentation of preliminary results was conducted at two scientific meetings while the research was being conducted. In 2010, a poster entitled: *Identifying Sources of Pathogen Contamination in Upper Fish River* was presented by University of West Alabama professor and project collaborator Dr. Brian Burnes at the Alabama Water

Resources Conference in Orange Beach, AL. At the same conference in 2011, an oral presentation: *Identifying Sources of Pathogen Contamination in Upper Fish River, Baldwin County, Alabama* containing information on the current state of the project was given by Dr. Burnes. As reported to the project manager by Dr. Burnes, the 2011 presentation was well received and other potential collaborations were cultivated.

Upper Fish River Source Tracking Project:
Tables

Table 1. Antibiotics used in E. coli growth assays.

Antibiotic	Acronym	Concentration (ug/ml)
Ampicilin	AMP	10
Amoxicillin	AMC	30
Chloramphenicol	CHL	30
Ciprofloxacin	CIP	5
Erythromycin	ERY	15
Gentamycin	GEN	10
Sulfisoxazole	GM	2
Neomycin	NEO	30
Nalidixic Acid	NAA	30
Streptomycin	STR	10
Spectinomycin	SPT	100
Oxytetracycline	OXY	30
Tetracycline	TET	30

Table 2. Historic *E. coli* counts and USGS precipitation data for Fish River at Woodhaven Dairy Rd.

Sample Date	E.coli/100ml MEAN	USGS[1] Discharge (cfs)	USGS[1] Rainfall (in)	3-Day USGS[1,2] Discharge (cfs)	3-Day USGS[1,2] Rainfall (in)
2007					
1/27	22	72 [A]	0.65 [A]	87 [A]	0.06 [A]
2/25	0	60 [A]	0.15 [A]	63 [A]	0.00 [A]
3/25	33	48 [A]	0.00 [A]	49 [A]	0.00 [A]
4/22	33	44 [A]	0.00 [A]	50 [A]	0.00 [A]
5/20	22	40 [A]	0.00 [A]	40 [A]	0.00 [A]
6/17	78	49 [A]	0.00 [A]	50 [A]	0.00 [A]
7/14	33	47 [A]	0.11 [A]	51 [A]	1.29 [A]
8/11	56	43 [A]	0.00 [A]	45 [A]	0.03 [A]
9/8	33	53 [A]	0.00 [A]	56 [A]	0.28 [A]
10/7	33	42 [A]	0.01 [A]	41 [A]	0.16 [A]
11/3	56	43 [A]	0.00 [A]	45 [A]	0.00 [A]
2008					
1/27	1833	136 [A]	0.00 [A]	136 [A]	2.53 [e A]
2/29	222	66 [A]	0.00 [A]	88 [A]	0.16 [A]
3/22	22	64 [A]	0.00 [A]	69 [A]	0.40 [A]
4/22	22	68 [A]	0.00 [A]	88 [A]	0.36 [A]
5/18	178	95 [A]	0.00 [A]	398 [A]	1.88 [A]
6/15	156	94 [A]	0.00 [A]	94 [A]	3.87 [A]
7/13	67	113 [A]	No Data	113 [A]	No Data
8/24	0	70 [A]	0.40 [A]	70 [A]	0.21 [A]
9/21	44	87 [A]	0.00 [A]	89 [A]	1.41 [A]
10/5	56	70 [P]	0.00 [P]	70 [P]	0.00 [P]
11/30	256	147 [P]	0.15 [P]	147 [P]	1.70 [P]
12/28	33	73 [P]	0.00 [P]	90 [P]	0.04 [P]
2009					
1/8	400	94 [P]	0.00 [P]	124 [P]	1.01 [P]
1/18	0	70 [P]	0.15 [P]	70 [P]	0.00 [P]
2/15	1300	217 [P]	0.02 [P]	333 [P]	4.64 [P]
4/11	33	84 [P]	0.00 [P]	90 [P]	0.00 [P]
5/10	11	71 [P]	0.00 [P]	83 [P]	0.00 [P]

[1] Explanation

[A] Approved for publication -- Processing and review completed.

[P] Provisional data subject to revision.

[e] Value has been estimated.

[2] USGS disharge maximum and accumulated precipitation within 3 days prior to sampling date.

Table 3. *E. coli* counts in Fish River in response to selected rainfall events.

Sample Date	Sample Time	E.coli/100ml MEAN	Rain Date	Volunteer Precipitation (in)
2008				
			10/17	0
10/18	8:45	11	10/18	0.15
10/18	17:45	22		
10/19	8:45	0	10/19	0
10/19	17:45	11		
10/20	9:15	11	10/20	0
10/20	18:15	0		
10/21	8:15	0	10/21	0
10/21	17:45	44		
10/22	8:30	78	10/22	0
10/22	19:15	33		**Total Rain=0.15**
			10/23	0
10/24	8:45	400	10/24	2.5
10/24	17:45	689		
10/25	7:45	422	10/25	0.03
10/25	17:45	200		
10/26	8:15	33	10/26	0
10/26	18:15	11		
10/27	8:45	33	10/27	0
10/27	17:45	22		
10/28	9:15	0	10/28	0
10/28	18:30	0		**Total Rain=2.53**
			11/7	0
11/8	9:15	122	11/8	0.69
11/8	17:15	44		
11/9	8:45	56	11/9	0
11/9	18:15	22		
11/10	9:15	0	11/10	No Data
11/10	18:15	22		
11/11	9:15	0	11/11	0
11/11	16:30	11		
11/12	9:00	0	11/12	0
11/12	15:45	178		**Total Rain=0.69**
			11/24	0
11/25	8:30	56	11/25	0.32
11/25	17:30	11		
11/26	8:30	11	11/26	0.02
11/26	16:00	11		
11/27	7:45	0	11/27	Trace
11/27	0:00	22		
11/28	8:30	0	11/28	0.02
11/28	17:00	44		
11/29	8:45	11	11/29	0.26
11/29	16:00	0		
11/30	7:30	156	11/30	1.6
11/30	14:30	11		**Total Rain=2.22**

Table 2 (Continued). *E. coli* counts in Fish River in response to selected rainfall events.

Sample Date	Sample Time	E.coli/100ml MEAN	Rain Date	Volunteer Precipitation (in)
2008				
			12/3	0
12/4	11:00	22	12/4	0.02
12/4	18:00	22		
12/5	8:30	22	12/5	0.32
12/5	16:30	33		
12/6	8:00	22	12/6	0
12/6	16:00	22		
12/7	8:45	0	12/7	0
12/7	16:30	11		
12/8	8:30	0	12/8	0
12/8	17:00	0		**Total Rain=0.34**
2009				
			3/14	0
3/15	8:30	22	3/15	1.1
3/16	8:30	1667	3/16	2.55
3/17	8:30	1389	3/17	0.85
3/18	8:30	67	3/18	0.02
3/19	8:30	33	3/19	Trace
				Total Rain=4.52
			4/12	0
4/13	8:30	78	4/13	0.04
4/14	8:30	2722	4/14	0.78
4/15	8:30	311	4/15	0.02
4/16	8:30	0	4/16	0
4/17	8:30	0	4/17	0
			4/18	0
				Total Rain=0.84
			5/3	0
			5/4	0.08
5/5	8:30	2256	5/5	4.4
5/6	8:30	133	5/6	0
5/7	8:30	89	5/7	Trace
5/8	8:30	44	5/8	0
5/9	8:30	11	5/9	0
				Total Rain=4.48

Table 3. *E. coli* counts at Fish River sites: Winter high water sampling event.

Location	Date	Time	Sample Amount (ml)	E coli colonies/ Sample Amount	E coli/ 100ml	Geomean/ 100ml
Woodhaven Dairy Road	2/14/2009	0905	1	15	1500	1482
			1	13	1300	
			2	32	1600	
			2	31	1550	
			3	44	1496	
			3	43	1462	
CR48/ Bohemain Park	2/14/2009	0845	1	10	1000	1350
			1	9	900	
			2	28	1400	
			2	37	1850	
			3	44	1496	
			3	51	1734	
CR54	2/14/2009	0820	1	19	1900	1555
			1	18	1800	
			2	30	1500	
			2	23	1150	
			3	45	1530	
			3	46	1564	
CR64, 30m downstream	2/14/2009	0800	1	3	300	327
			1	1	100	
			2	9	450	
			2	6	300	
			3	26	884	
			3	10	340	
US90	2/14/2009	0744	1	5	500	635
			1	4	400	
			2	18	900	
			2	10	500	
			3	21	714	
			3	30	1020	
Interstate 10	2/14/2009	0730	1	1	100	199
			1	2	200	
			2	3	150	
			2	5	250	
			3	6	204	
			3	12	408	

Table 4. *E. coli* counts at Fish River sites: Spring low water sampling event.

Location	Date	Time	Sample Amount (ml)	E coli colonies/ Sample Amount	E coli/ 100ml	Mean[1]/ 100ml
Woodhaven Dairy Road	4/27/2009	0740	4	1	25	10
			4	0	0	
			4	1	25	
			4	0	0	
			4	0	0	
CR48/ Bohemain Park	4/27/2009	0730	4	1	25	30
			4	2	50	
			4	1	25	
			4	2	50	
			4	0	0	
CR54	4/27/2009	0800	4	2	50	20
			4	0	0	
			4	0	0	
			4	2	50	
			4	0	0	
CR64, 30m downstream	4/27/2009	0825	4	0	0	30
			4	1	25	
			4	0	0	
			4	3	75	
			4	2	50	
US90	4/27/2009	0839	4	0	0	15
			4	0	0	
			4	2	50	
			4	1	25	
			4	0	0	
Interstate 10	4/27/2009	0855	4	0	0	15
			4	1	25	
			4	1	25	
			4	1	25	
			4	0	0	

[1] Geometric Mean fails to calculate a number when zeros present in series.
Arithmetic Mean calculated.

Table 5. *E. coli* counts at Fish River sites: Spring high water sampling event.

Location	Date	Time	Sample Amount (ml)	E coli colonies/ Sample Amount	E coli/ 100ml	Geomean/ 100ml	Mean/ 100ml
Woodhaven Dairy Road	5/4/2009	1759	2	49	2450	2447	2450
			2	52	2600		
			2	46	2300		
CR48/ Bohemain Park	5/4/2009	1750	2	124	6200	5878	5883
			2	117	5850		
			2	112	5600		
CR54	5/4/2009	1735	2	115	5750	5201	5217
			2	95	4750		
			2	103	5150		
CR64, 30m downstream	5/4/2009	1715	2	58	2900	3015	3017
			2	63	3150		
			2	60	3000		
US90	5/4/2009	1705	2	117	5850	5698	5700
			2	115	5750		
			2	110	5500		
Interstate 10	5/4/2009	0654	2	16	800	1132	1167
			2	29	1450		
			2	25	1250		

Table 6. *E. coli* counts for samplings events submitted for antibiotic resistance analysis

		Sampling Locations					
		Interstate 10	US 90	CR 64	CR 54	CR 48 Bohemian Park	Woodhaven Dairy Road
Date	Condition	E. coli/100ml[1]					
1/4/2009	Rain	97	380	125	490	129	310
1/28/2009	Dry	0	2[2]	0	1[2]	1[2]	1[2]
3/1/2009	Rain	1833	2600	3833	3533	3333	3433
11/4/2010	Rain	1733	1600	1266	1800	2733	1500
11/18/2010	Rain	766	1200	1033	1000	1100	1366
1/1/2011	Rain	169	261	269	169	269	69

[1] Arithmatic mean calculated for each data set.

[2] Only five E. coli colonies were detected; the number reflects total colonies not E. coli/100ml

Table 7. Classification of *E. coli* from known sources in the Upper Fish River watershed.

		Source						
Date	**Classification**	**Human**	**%**	**Bovine**	**%**	**Equine**	**%**	**Total**
05/01/09	Human	22	71.0	11	19.3	5	16.7	38
	Bovine	5	16.1	34	59.6	4	13.3	43
	Equine	4	12.9	12	21.1	21	70.0	37
	Total	31	100	57	100	30	100	118
08/19/10	Human	11	52.4	8	23.5	2	5.7	21
	Bovine	9	42.9	17	50.0	9	25.7	35
	Equine	1	4.8	9	26.5	24	68.6	34
	Total	21	100	34	100	35	100	90

Table 8. Classification of *E. coli* from the Upper Fish River[a].

		Sample													
Date	**Class**	**I-10**	**%**	**US 90**	**%**	**CR 64**	**%**	**CR 54**	**%**	**CR 48**	**%**	**WHD**	**%**	**Totals**	**%**
Jan 2009	Bovine	0.0	0.0	253.3	66.7	0.0	0.0	98.0	20.0	0.0	0.0	62.0	20.0	500.0	24.6
	Equine	97.0	100.0	126.7	33.3	0.0	0.0	359.3	73.3	64.5	50.0	186.0	60.0	1090.2	53.7
	Human	0.0	0.0	0.0	0.0	125.0	100.0	32.7	6.7	64.5	50.0	62.0	20.0	440.8	21.7
Feb 2009	Bovine	33.7	16.7	467.5	69.6	351.1	88.9	1070.3	68.0	271.4	19.4	325.8	22.0	2782.3	44.7
	Equine	168.3	83.3	146.1	21.7	43.9	11.1	314.8	20.0	1047.0	75.0	832.5	56.1	2763.8	44.4
	Human	0.0	0.0	58.4	8.7	0.0	0.0	188.9	12.0	77.6	5.6	325.8	22.0	676.9	10.9
Mar 2009	Bovine	6.4	42.9	8.8	56.0	15.0	33.3	6.7	***33.3***	***10.0***	***33.3***	***3.3***	***33.3***	249.0	39.2
	Equine	7.5	50.0	4.4	28.0	30.0	66.7	6.7	***33.3***	***10.0***	***33.3***	***3.3***	***33.3***	273.2	43.0
	Human	1.1	7.1	2.5	16.0	0.0	0.0	6.7	***33.3***	***10.0***	***33.3***	***3.3***	***33.3***	113.4	17.8
Apr 2009	Bovine	***1833.0***	***100.0***	***866.7***	***33.3***	***3833.0***	***100.0***	***0.0***	***0.0***	***3333.0***	***100.0***	***1144.3***	***33.3***	11343.4	59.5
	Equine	***0.0***	***0.0***	***866.7***	***33.3***	***0.0***	***0.0***	***3533.0***	***100.0***	***0.0***	***0.0***	***1144.3***	***33.3***	5677.4	29.8
	Human	***0.0***	***0.0***	***866.7***	***33.3***	***0.0***	***0.0***	***0.0***	***0.0***	***0.0***	***0.0***	***1144.3***	***33.3***	2044.4	10.7
May 2009	Bovine	233.4	20.0	***5700.0***	***100.0***	1580.3	52.4	3581.9	68.8	918.8	20.0	918.8	37.5	13194.2	58.3
	Equine	933.6	80.0	0.0	0.0	862.0	28.6	651.3	12.5	2206.1	48.0	1531.3	62.5	6353.3	28.1
	Human	0.0	0.0	0.0	0.0	574.7	19.0	976.9	18.8	1470.8	32.0	0.0	0.0	3092.1	13.7
1 Nov2010	Bovine	666.5	38.5	492.3	30.8	670.2	52.9	942.9	52.4	1366.5	50.0	***500.0***	***33.3***	4863.0	43.7
	Equine	666.5	38.5	492.3	30.8	223.4	17.6	600.0	33.3	455.5	16.7	***500.0***	***33.3***	3074.6	27.6
	Human	399.9	23.1	615.4	38.5	372.4	29.4	257.1	14.3	911.0	33.3	***500.0***	***33.3***	3194.4	28.7
2 Nov2010	Bovine	***255.3***	***33.3***	***400.0***	***33.3***	***344.3***	***33.3***	***333.3***	***33.3***	651.9	59.3	739.9	54.2	2917.4	41.9
	Equine	***255.3***	***33.3***	***400.0***	***33.3***	***344.3***	***33.3***	***333.3***	***33.3***	0.0	0.0	455.3	33.3	1921.7	27.6
	Human	***255.3***	***33.3***	***400.0***	***33.3***	***344.3***	***33.3***	***333.3***	***33.3***	448.1	40.7	170.8	12.5	2126.0	30.5
Jan 2011	Bovine	123.5	73.1	155.4	59.5	112.6	41.9	68.9	40.7	93.8	34.9	37.2	53.8	841.4	49.3
	Equine	45.5	26.9	99.4	38.1	150.1	55.8	100.1	59.3	175.2	65.1	31.8	46.2	847.4	49.7
	Human	0.0	0.0	6.2	2.4	6.3	2.3	0.0	0.0	0.0	0.0	0.0	0.0	17.2	1.0

[a] Numbers in italics are extrapolated from very limited data and should be regarded as such

Table 9. Results from Human *Enterococcus* and *Bacteroidetes* testing on Fish River water samples collected at US 90, CR54 and Woodhaven Dairy Rd.

		Sampling Locations		
Human Enterococcus		**US 90**	**CR 54**	**Woodhaven Dairy Rd**
Date Sampled	**Condition**	**Detected (✌) or Not Detected (X)**		
4/7/2010	**Baseflow**	**X**	**X**	**X**
5/17/2010	**Rain**	**X**	**X**	**✌**
1/1/2011	**Rain**	**X**	**✌**	**X**
Human Bacteroidetes				
Date Sampled	**Condition**	**Detected (✌) or Not Detected (X)**		
4/7/2010	**Baseflow**	**X**	**X**	**X**
5/17/2010	**Rain**	**X**	**X**	**X**
1/1/2011	**Rain**	**✌**	**✌**	**✌**

Table 10. Results from Bovine *Enterococcus* and *Bacteroidetes* testing on Fish River water samples collected at US 90, CR54 and Woodhaven Dairy Rd.

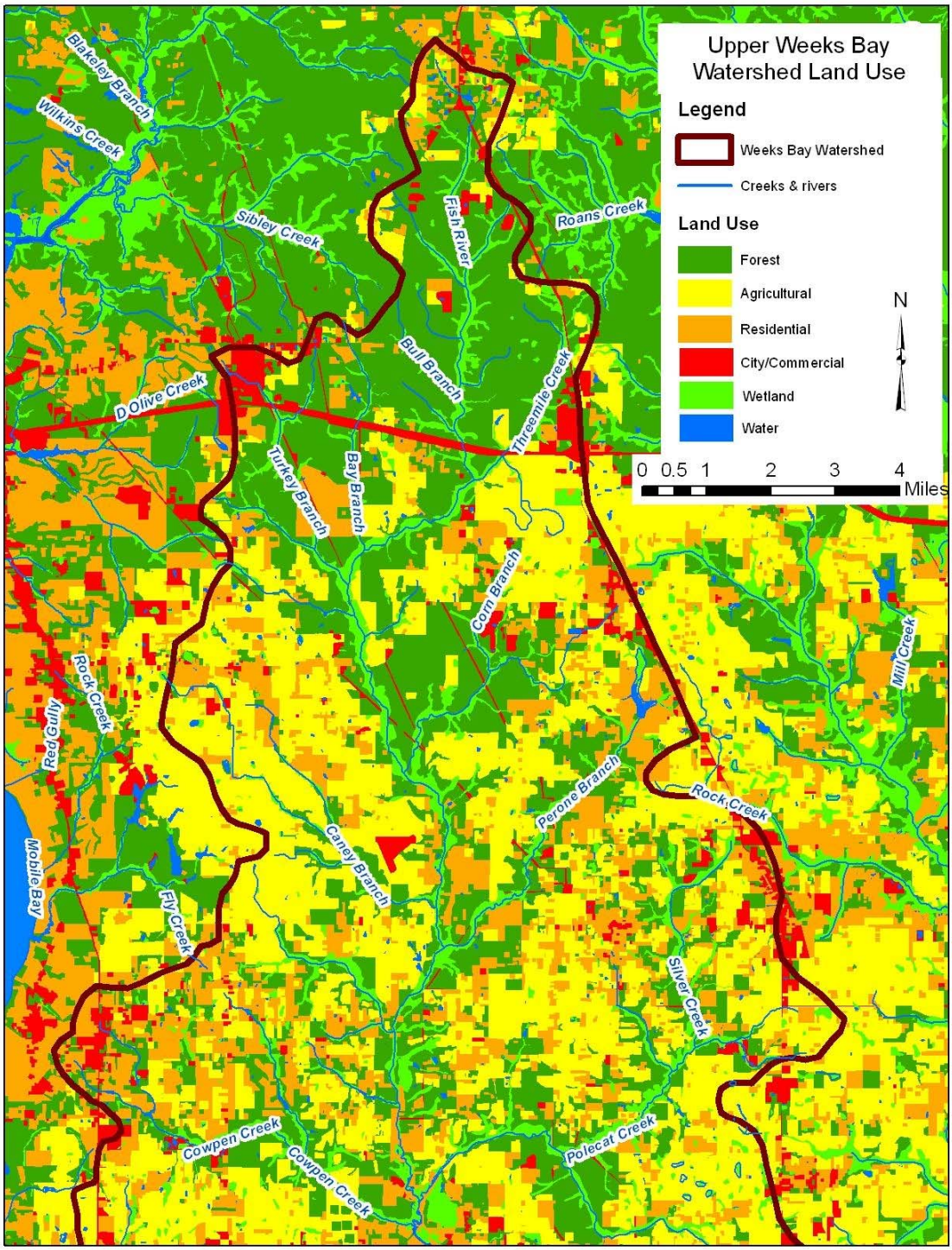
		Sampling Locations		
Bovine Enterococcus		**US 90**	**CR 54**	**Woodhaven Dairy Rd**
Date Sampled	**Condition**	**Detected (✓) or Not Detected (X)**		
4/7/2010	**Baseflow**	**X**	**✓**	**✓**
5/17/2010	**Rain**	**X**	**X**	**X**
1/1/2011	**Rain**	**X**	**X**	**X**
Bovine Bacteroidetes				
Date Sampled	**Condition**	**Detected (✓) or Not Detected (X)**		
4/7/2010	**Baseflow**	**X**	**X**	**X**
5/17/2010	**Rain**	**X**	**X**	**X**
1/1/2011	**Rain**	**X**	**X**	**X**

Table 11. Results from Equine *Bacteroidetes* testing on Fish River water samples collected at US 90, CR54 and Woodhaven Dairy Rd.

Equine Bacteroidetes		Sampling Locations		
		US 90	CR 54	Woodhaven Dairy Rd
Date Sampled	Condition	Detected (✓) or Not Detected (X)		
2/5/2011	Rain	✓	✓	✓
3/30/2011	Rain	X	✓	X

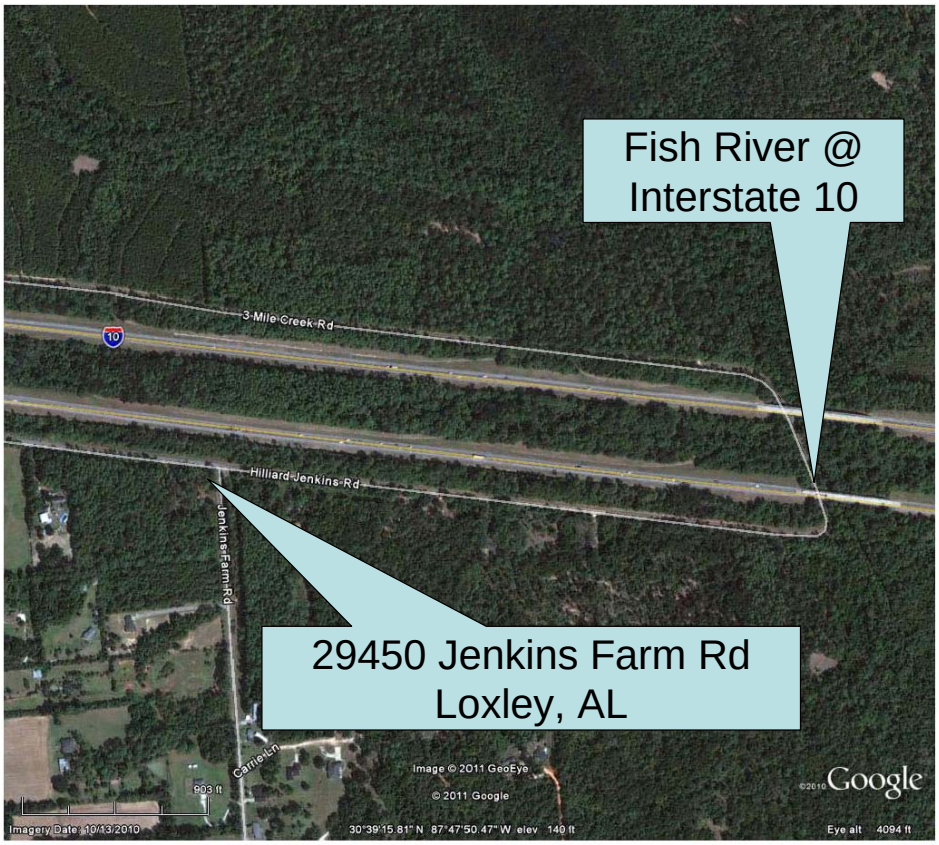
Upper Fish River Source Tracking Project:
Figures

Figure 1. Land use of the upper Fish River (Baldwin County Commission, 2005)



The watersheds of Cowpen Creek and Polecat Creek are not included in the study area.

Figure 2. Proximity of rainfall monitor location to Fish River water sampling site.



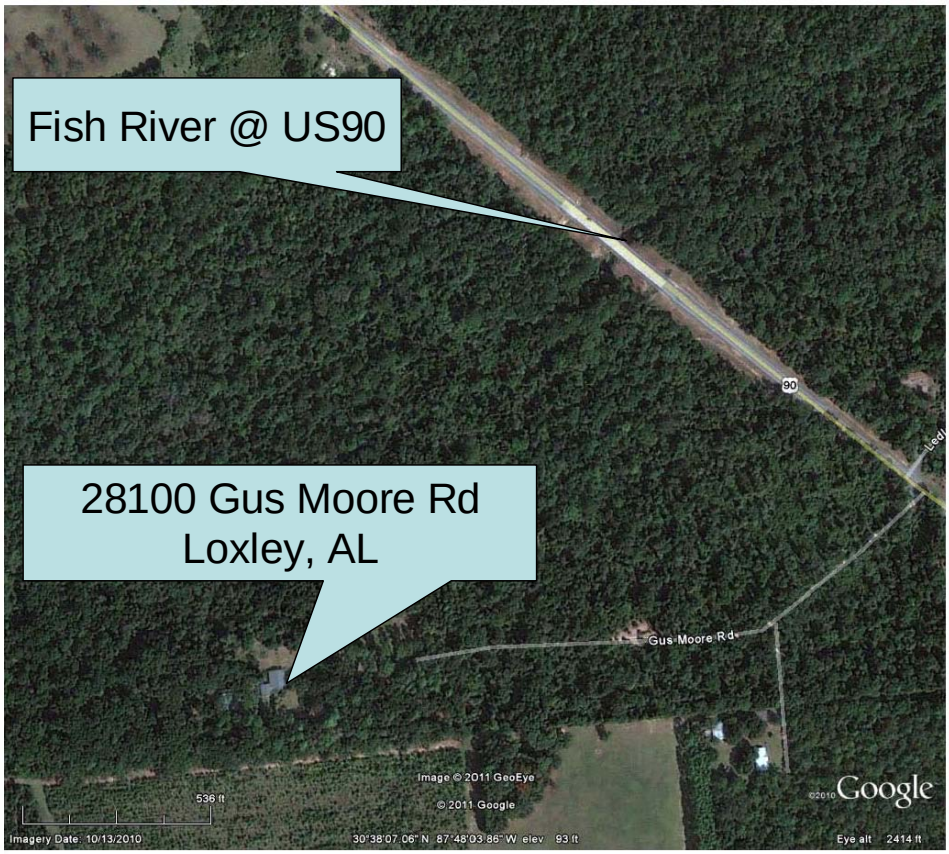
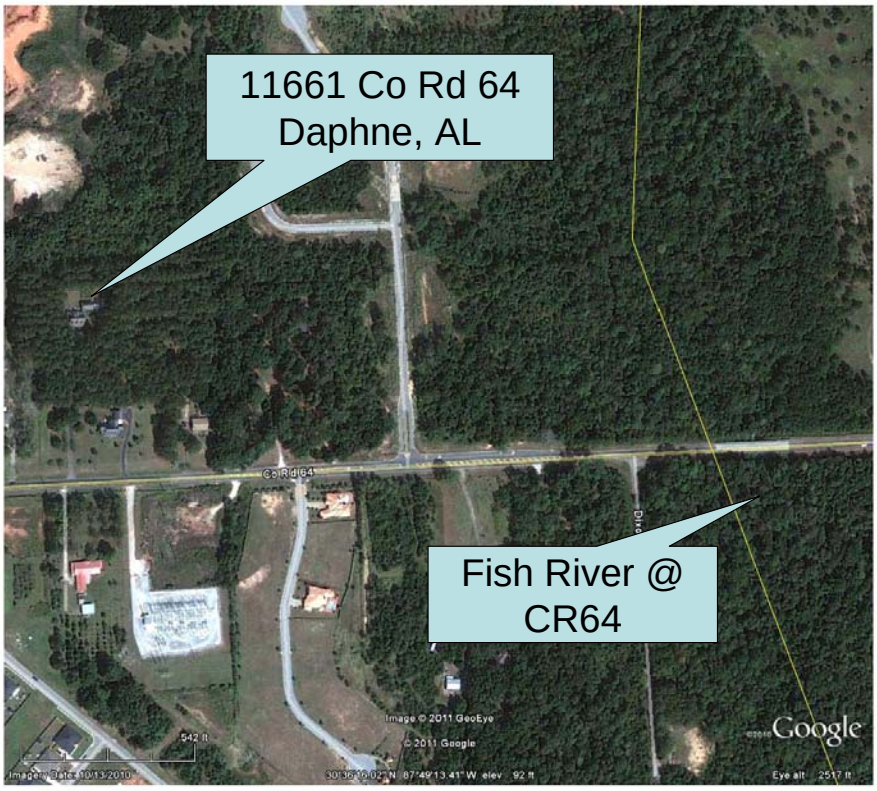


Figure 2 (continued). Proximity of rainfall monitor location to Fish River water sampling site.



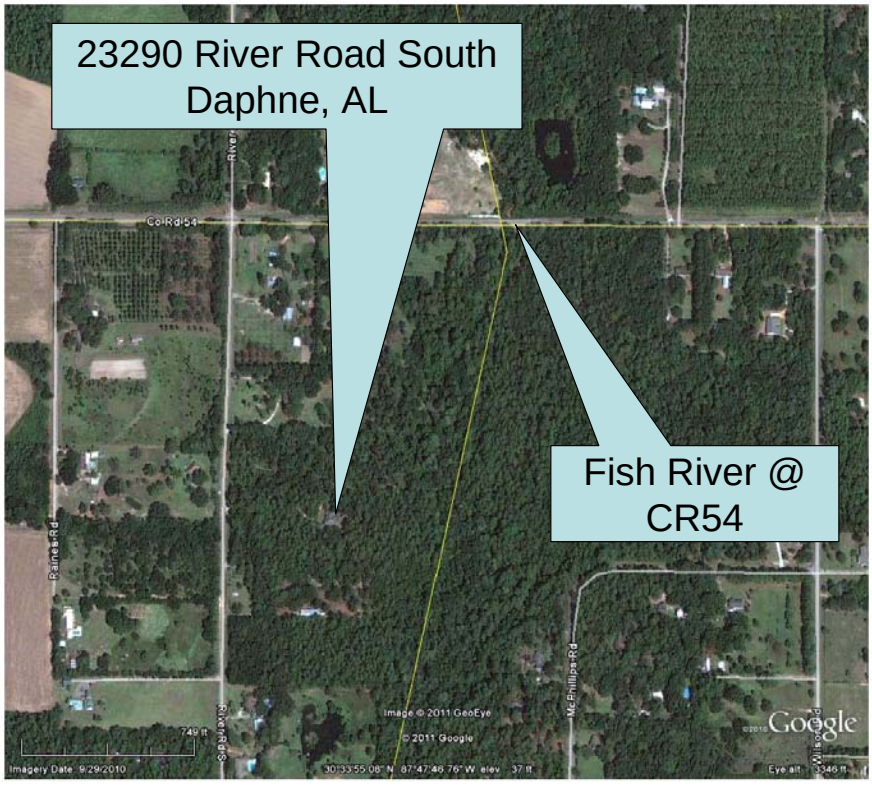
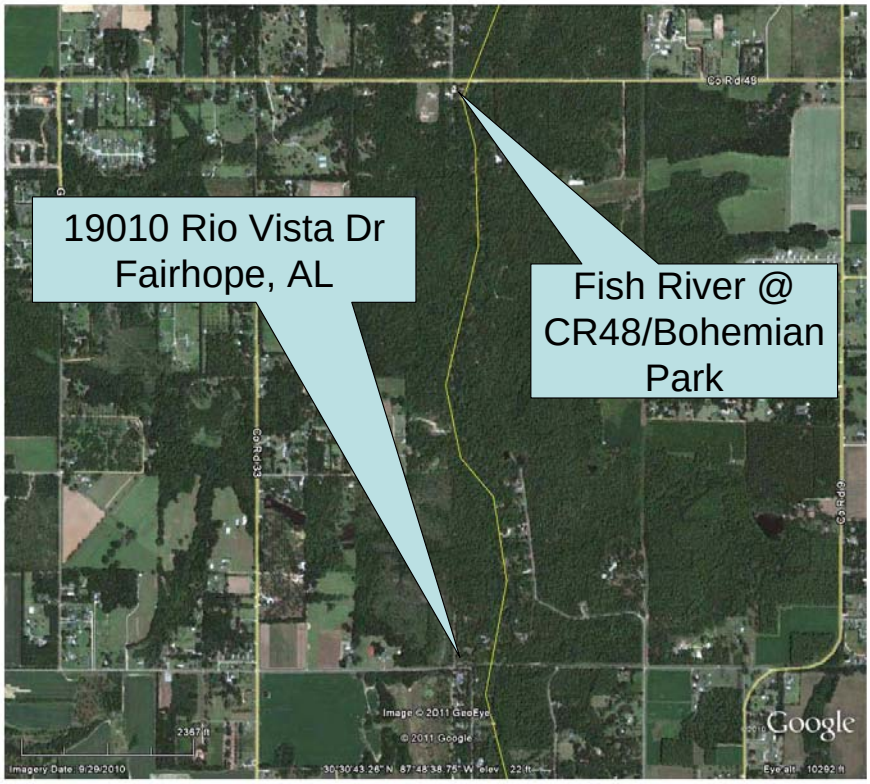


Figure 2 (continued). Proximity of rainfall monitor location to Fish River water sampling site.



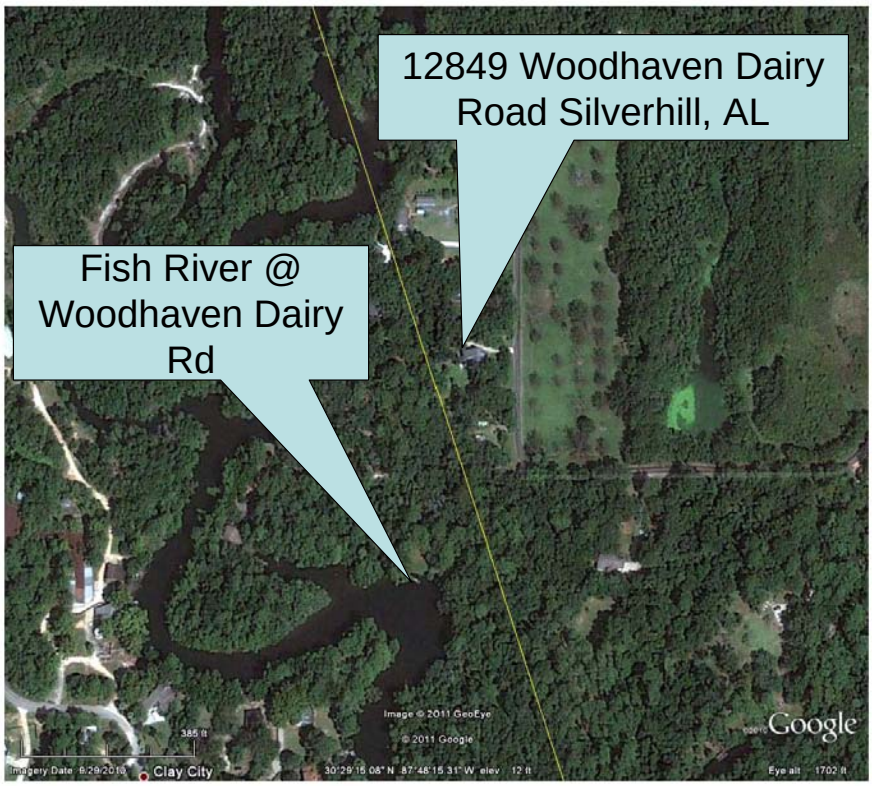
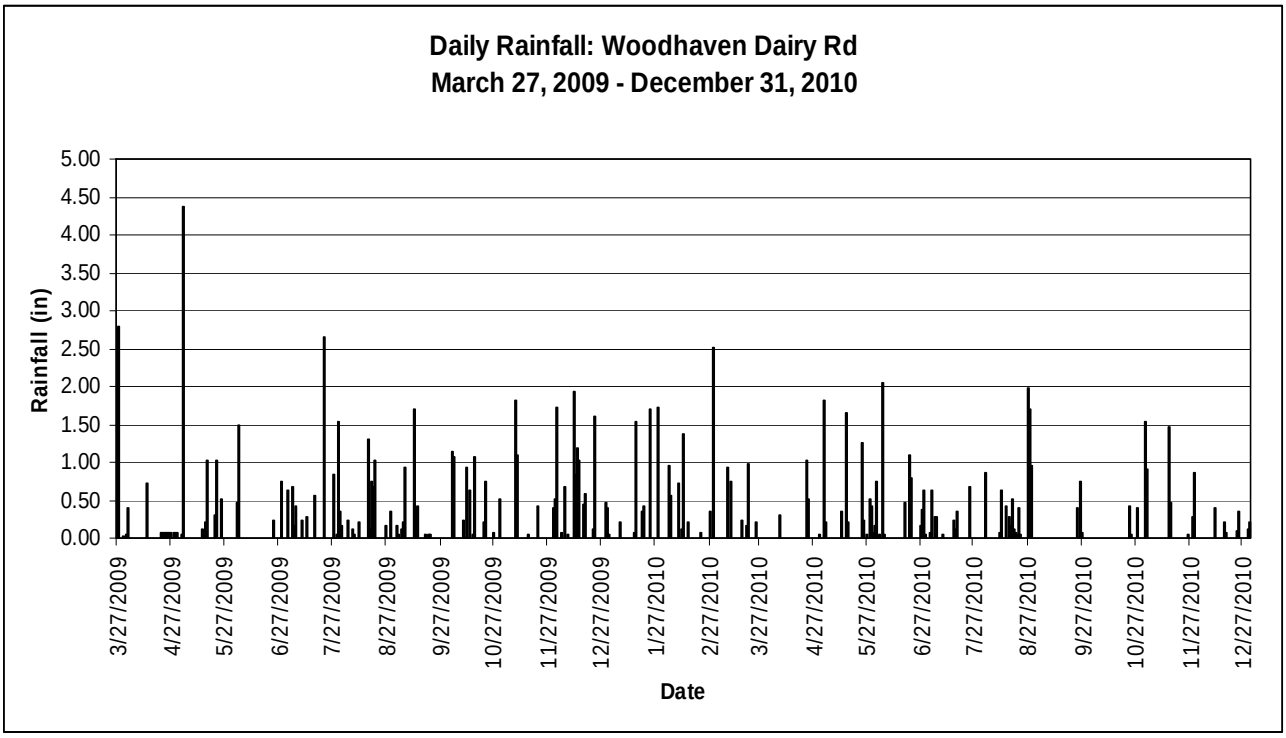


Figure 3. Daily rainfall amounts collected by volunteer monitors



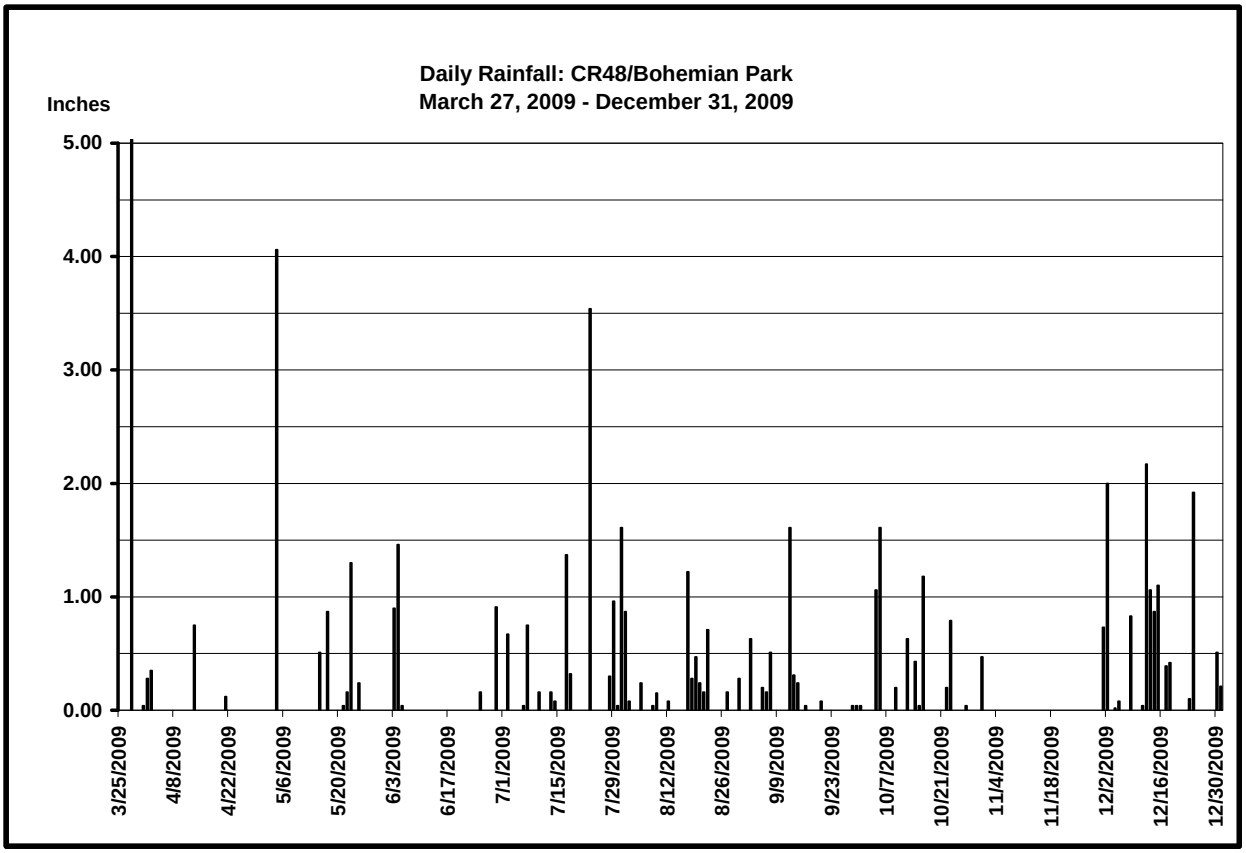
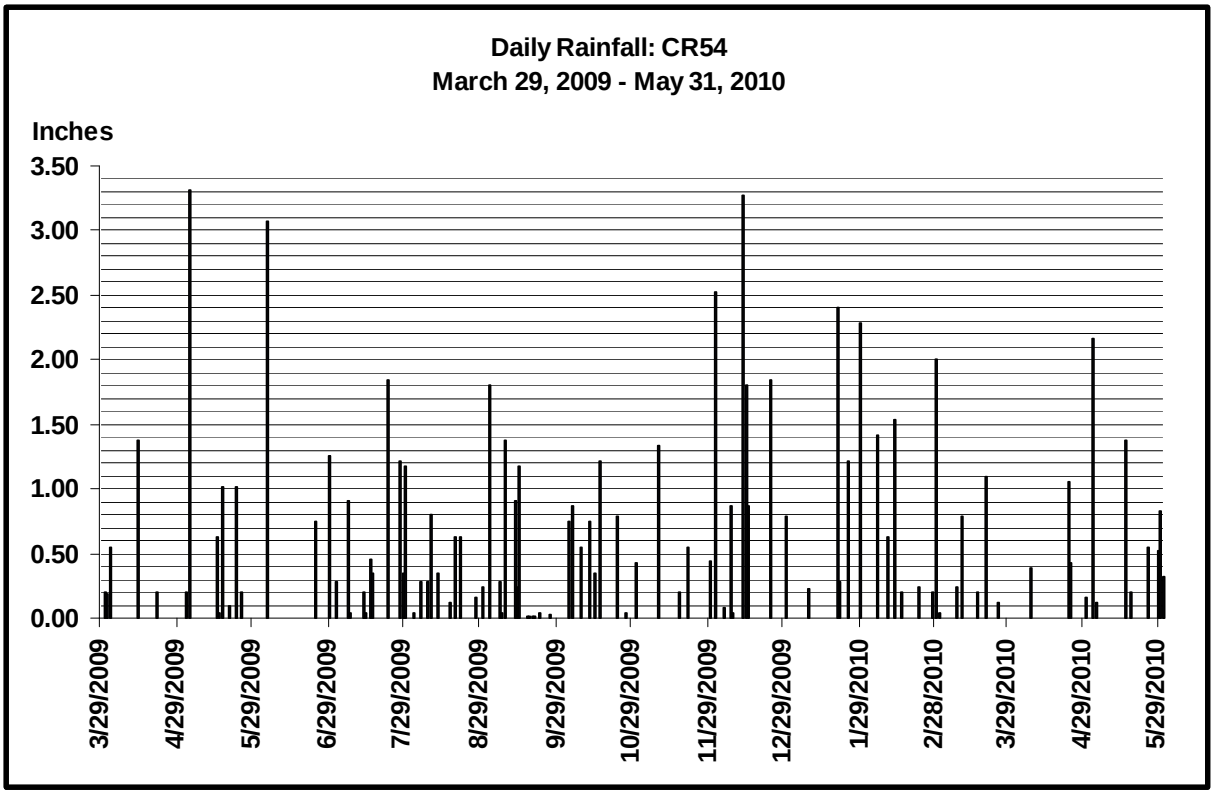


Figure 3 (Continued). Daily rainfall amounts collected by volunteer monitors



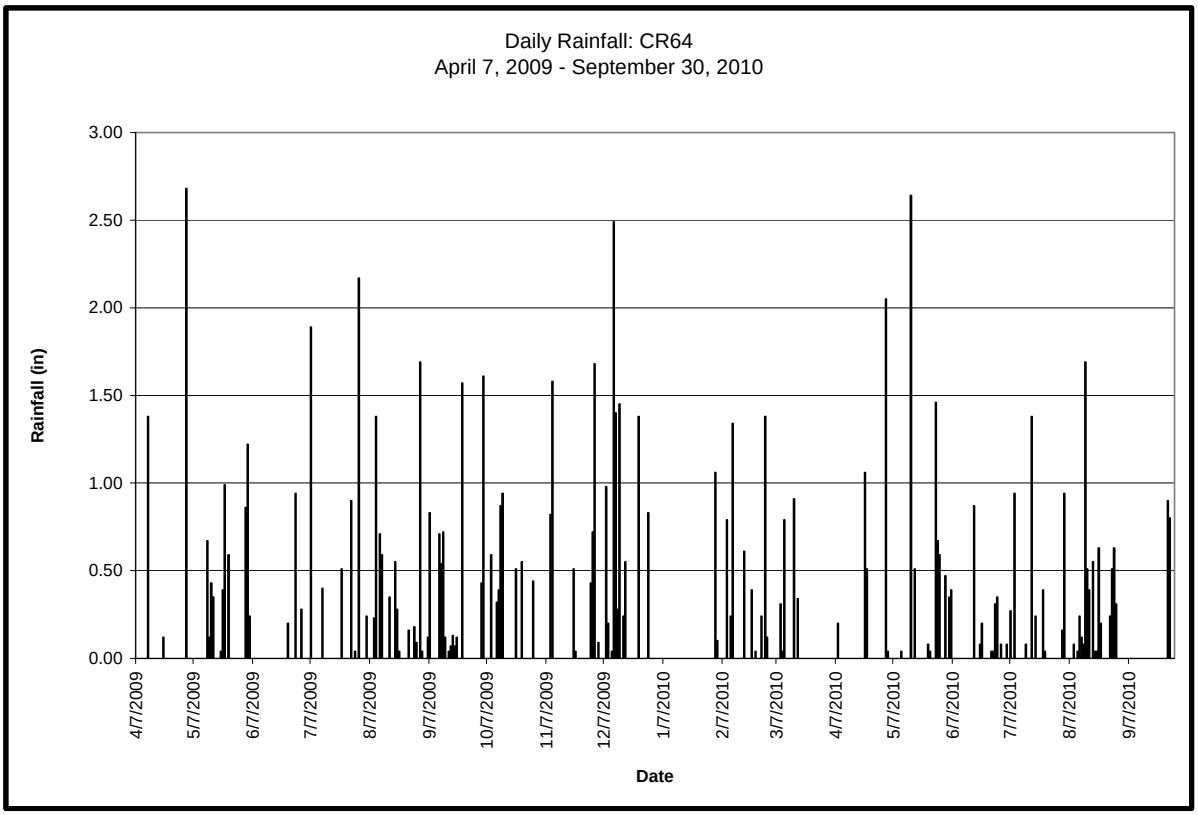


Figure 3 (Continued). Daily rainfall amounts collected by volunteer monitors

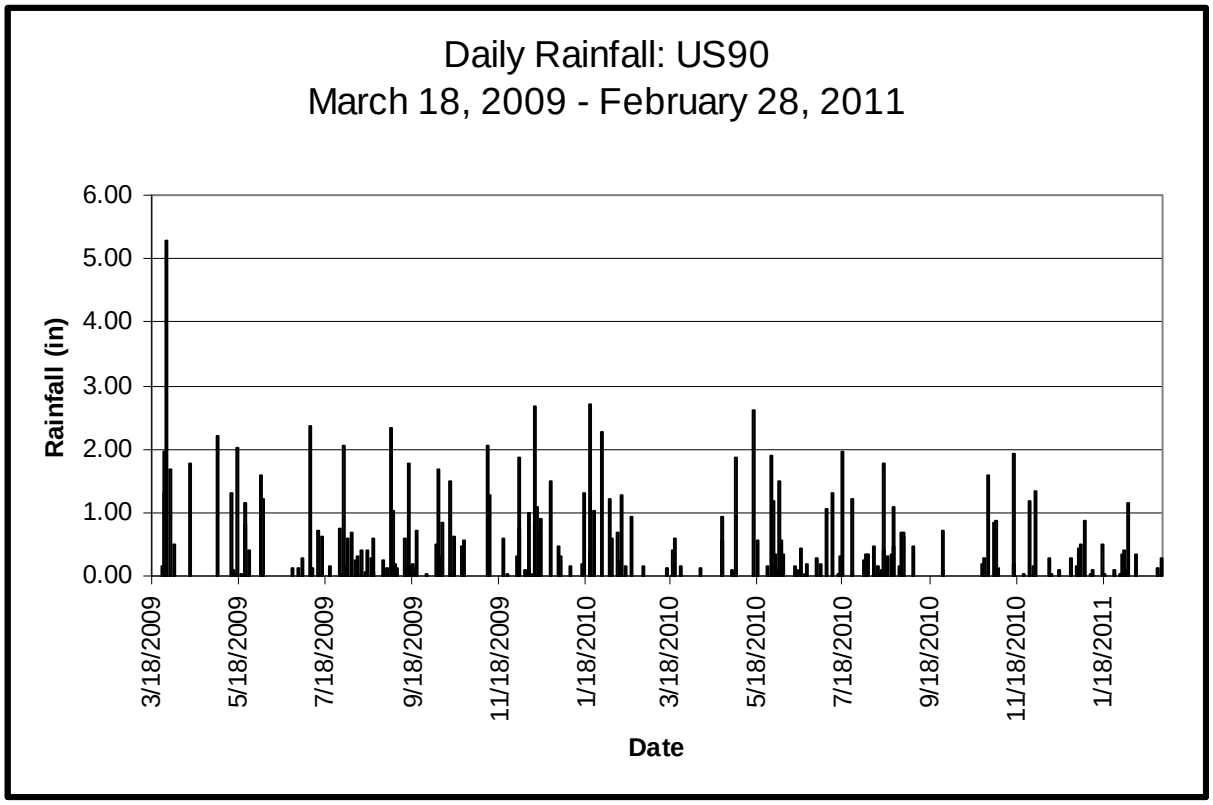


Figure 4. Upper Fish River sampling sites including latitude and longitude

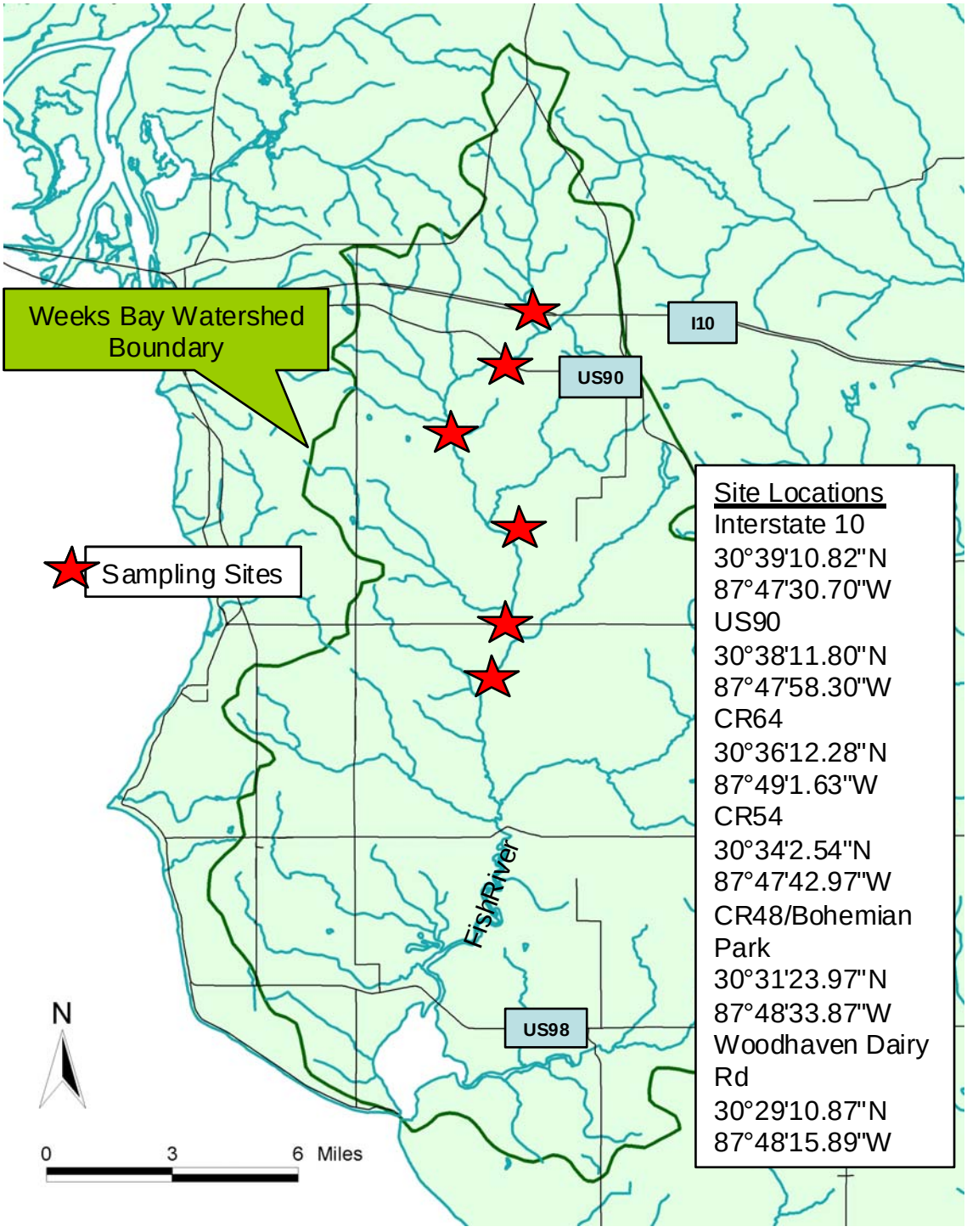


Figure 5. Sampling sites for known E. coli sources: Human, Bovine and Equine

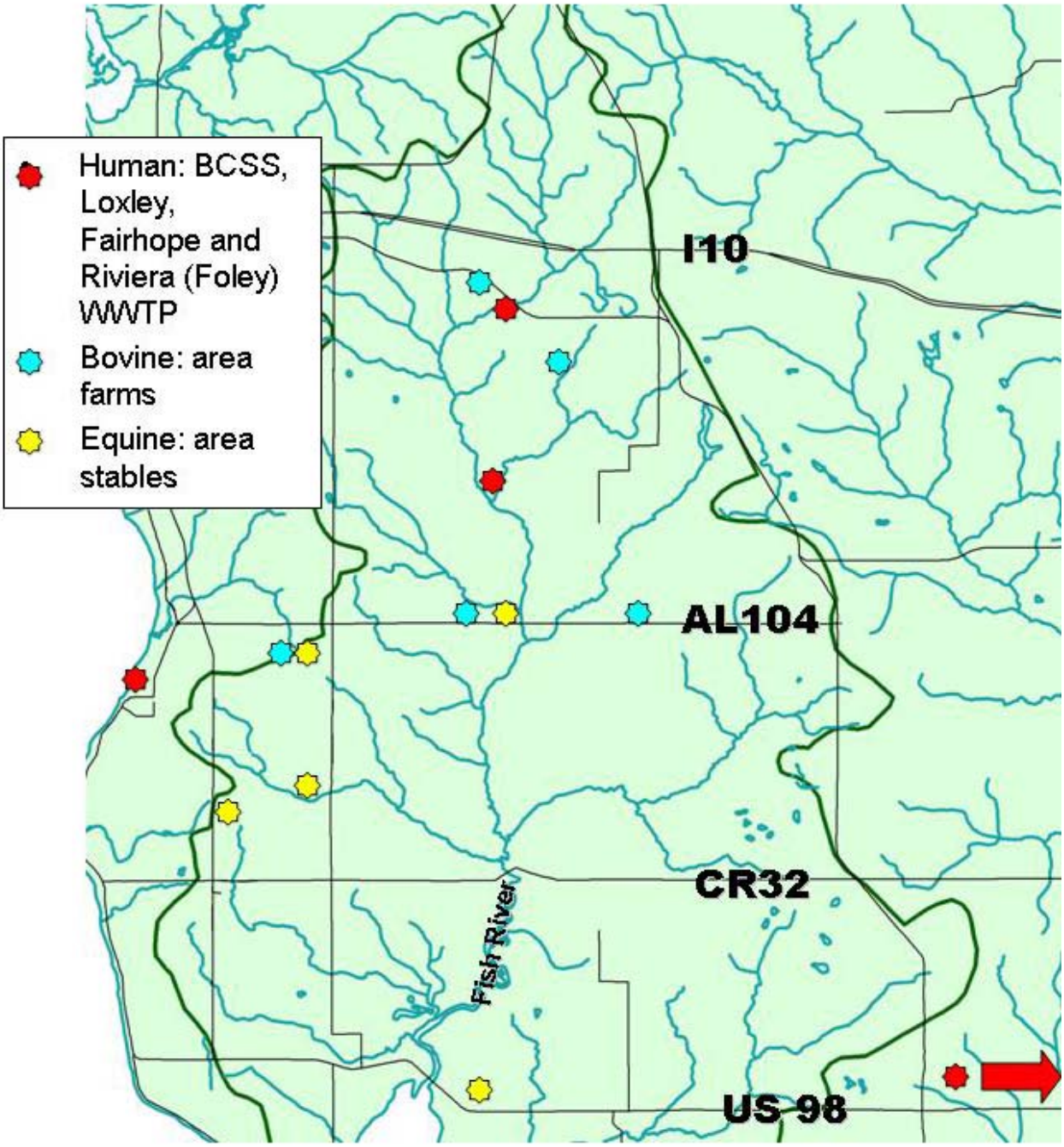


Figure 6. Discriminant Function Scores and Territory Map of *E. coli* from known sources in the Fish River watershed. Each point represents one *E. coli* isolate. The points are shaded by group: the lightest shading indicates human *E. coli*, the medium shading indicates bovine E. coli, and the darkest shading indicates equine *E. coli*.

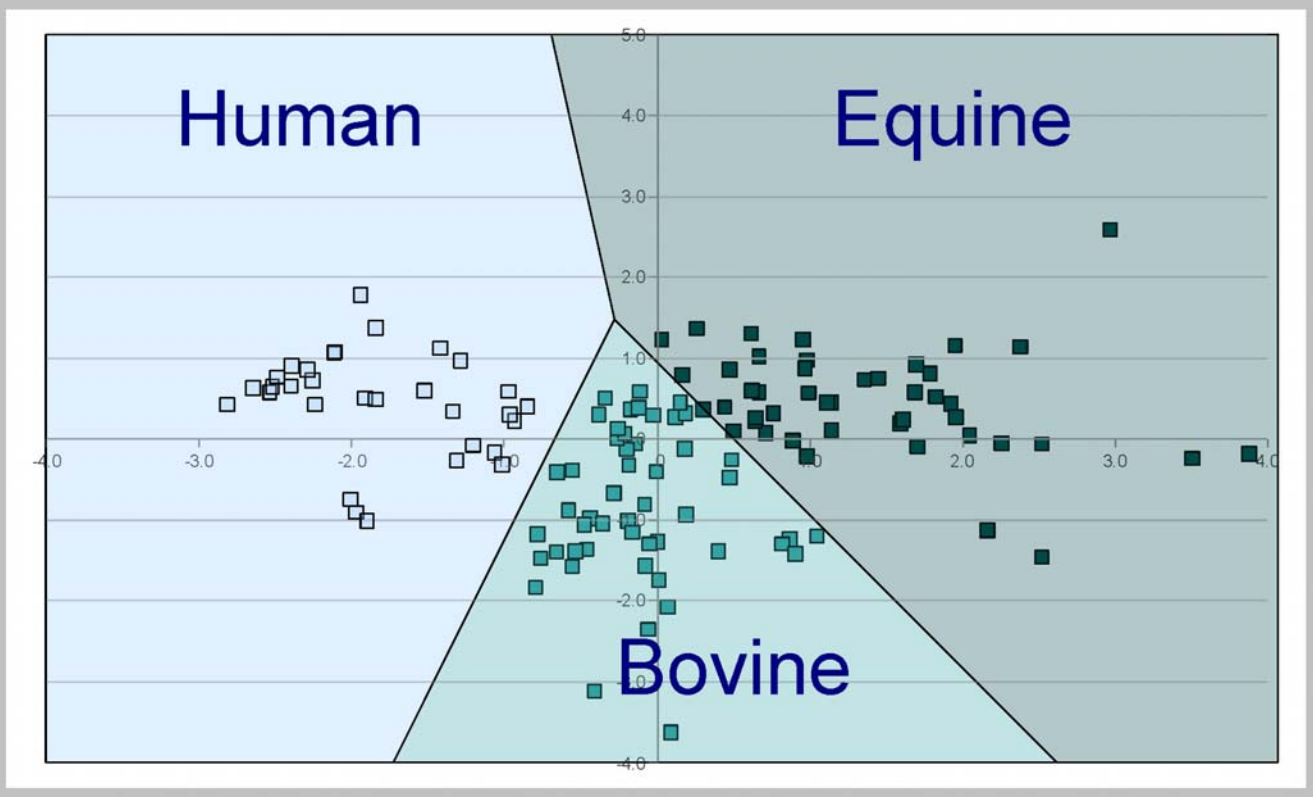


Figure 7. Discriminant Function Scores and Territory Map of *E. coli* from the Fish River. Each point represents one *E. coli* isolate. The points are shaded by group: the lightest shading indicates human E. coli, the medium shading indicates bovine *E. coli*, and the darkest shading indicates equine *E. coli*.

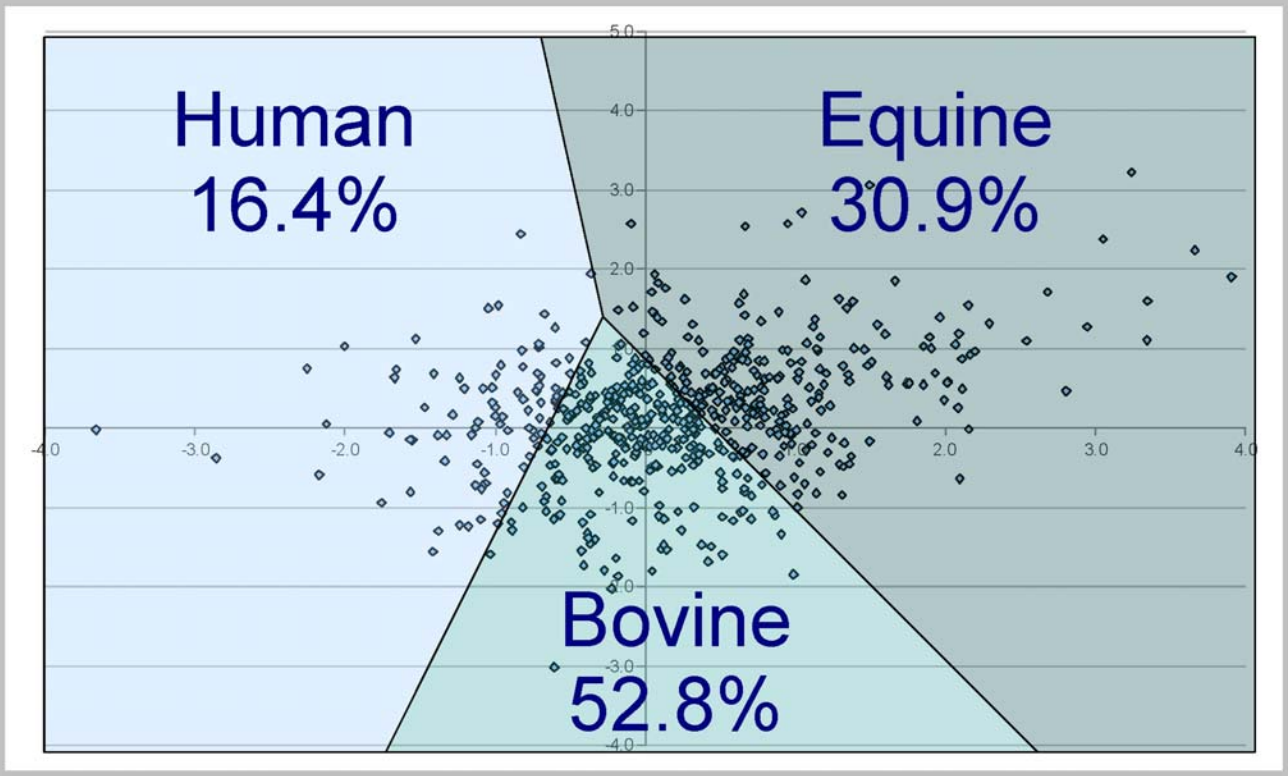


Figure 8. Classification of *E. coli* isolated from Fish River at each sampling event.

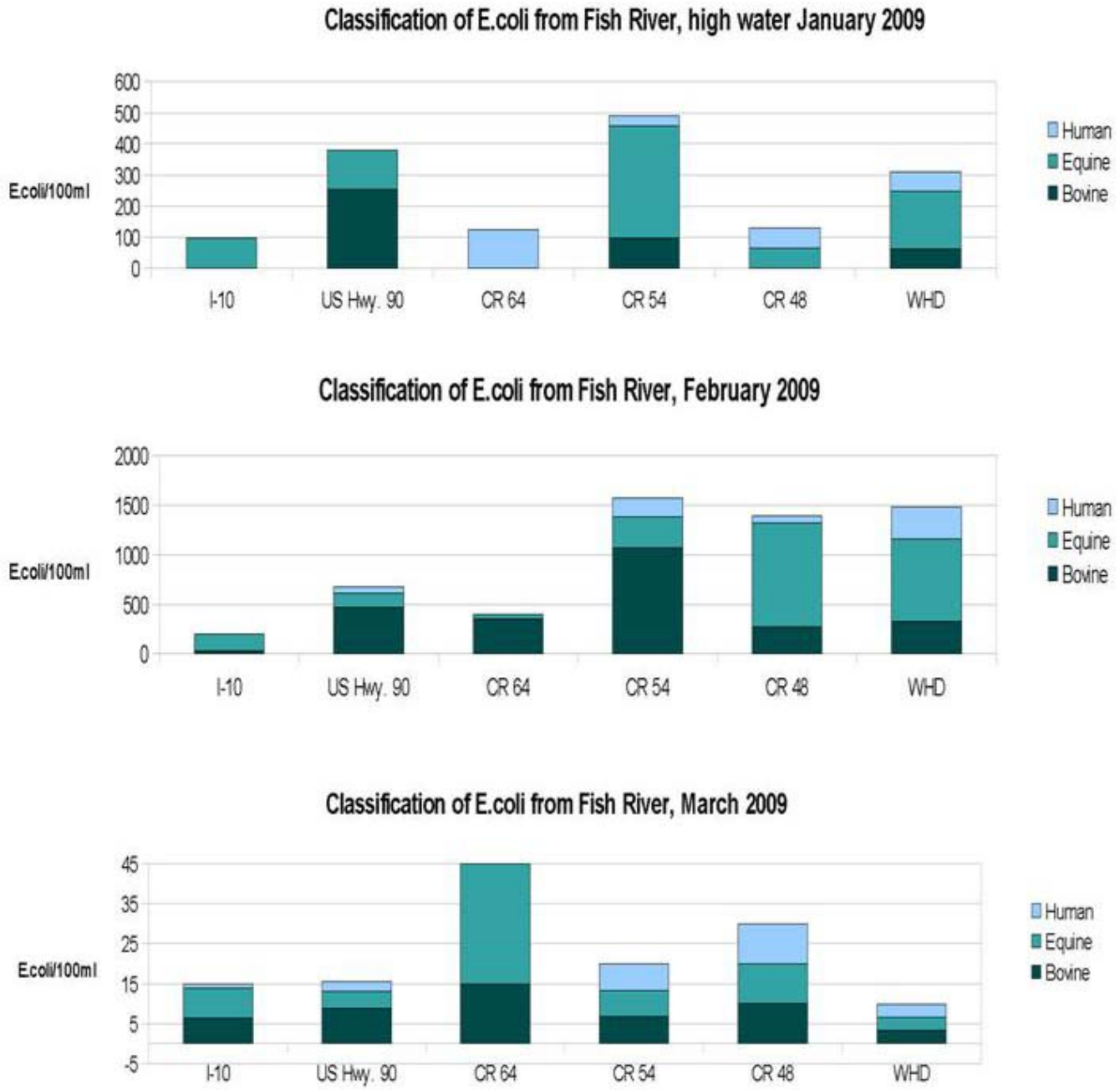


Figure 8 (continued). Classification of *E. coli* isolated from Fish River at each sampling event.

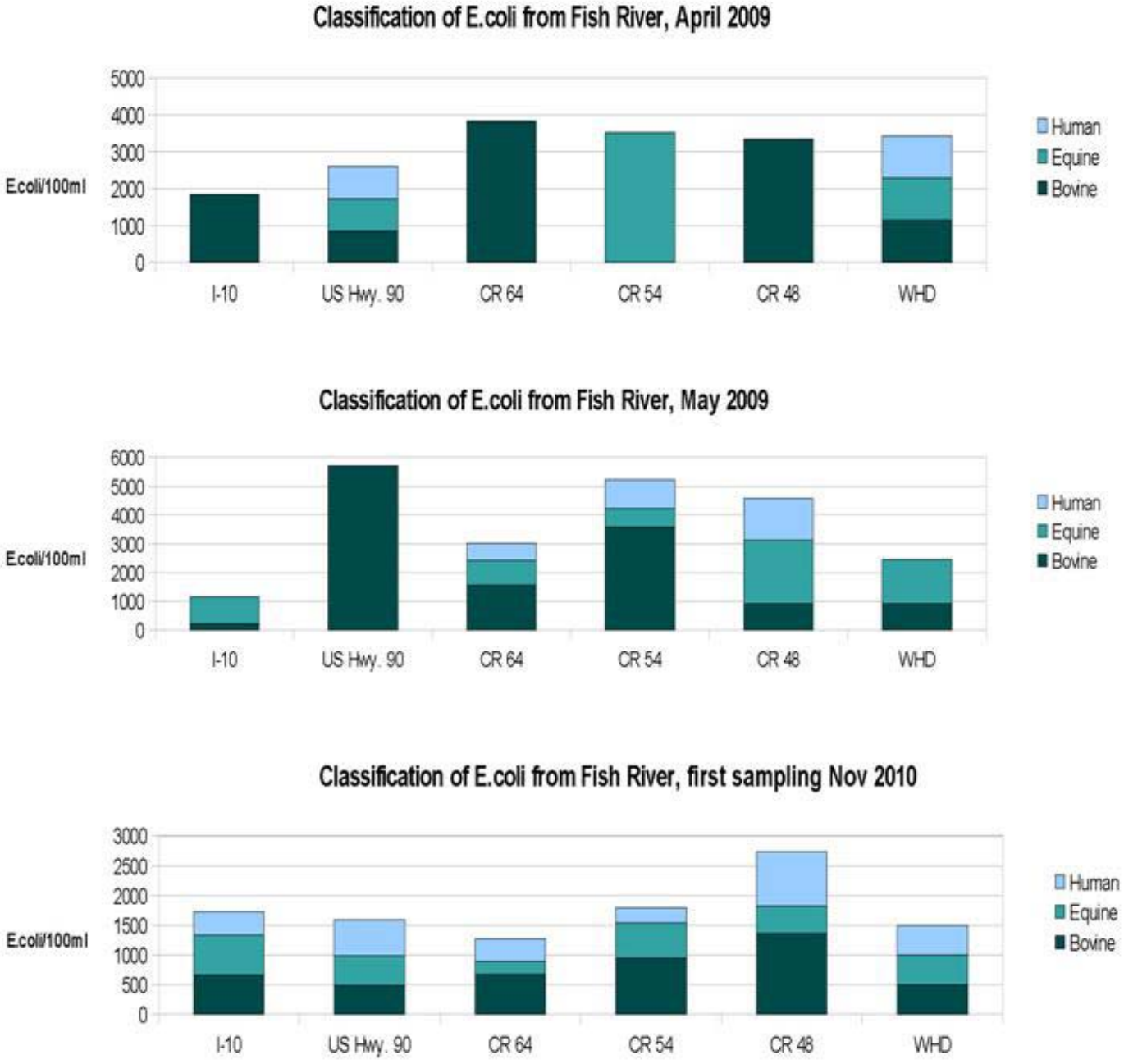
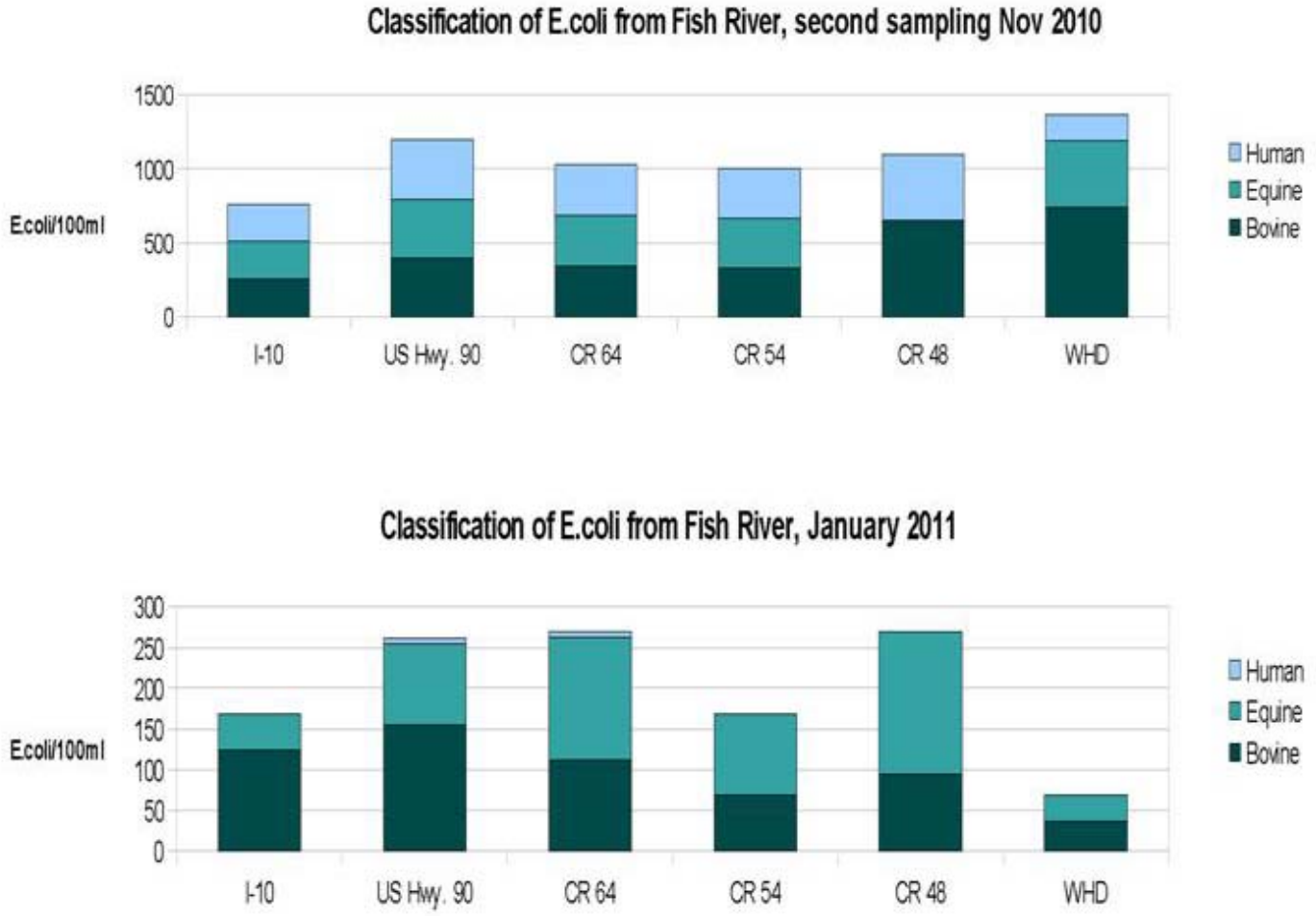


Figure 8 (continued). Classification of *E. coli* isolated from Fish River at each sampling event.



Final Budget Information and Expenditures

Proposal Budget

Budget Category	MBNEP EPA Funds Requested				Other Federal Funds	Non-Federal Matching Funds/In-Kind				Non-Federal Matching Funds/Cash				Total Project Value
Salaries (as Volunteer Time @ $18.77/hr)						$10,000								$10,000
Fringe														
Travel						$4,000								$4,000
Supplies	$7,000									$7,000				$14,000
Equipment	$3,000									$2,000				$5,000
Sub-Contractual														
Other	$12,000									$3,000				$15,000
Total	$22,000					$14,000				$12,000				$48,000

Actual Budget

Budget Category	MBNEP EPA Funds Requested				Other Federal Funds	Non-Federal Matching Funds/value				Non-Federal Matching Funds/Cash				Total Project Value
	Invoice #1	Invoice #2	Invoice #3	Total		Invoice #1	Invoice #2	Invoice #3	Total	Invoice #1	Invoice #2	Invoice #3	Total	
Administrative Time														
Value of Waiver of Admininstrative Time														$0.00
Volunteer Time 866 hr @ $18.77/hr														
Total Volunteer Time							$4,692.50	$11,562.32	$16,254.82					$16,254.82
Travel														
Supplies														
Antibiotic Resistance Testing/Shipping		$1,816.66	$385.34	$2,202.00							$783.91	$1,418.09	$2,202.00	$4,404.00
Equipment														
Sterilizer	$3,000.00			$3,000.00						$3,000.00			$3,000.00	$6,000.00
Sub-Contractual														
Other:														
PCR Analysis/Shipping		$6,437.65	$5,562.35	$12,000.00							$2,777.91	$2,925.53	$5,703.44	$17,703.44
Total	$3,000.00	$8,254.31	$5,947.69	$17,202.00	$0.00	$0.00	$4,692.50	$11,562.32	$16,254.82	$3,000.00	$3,561.82	$4,343.62	$10,905.44	$44,362.26

Appendix A

Coliscan Easygel Method Details

Coliscan Easygel
by Micrology Laboratories, Goshen, IN

Coliform bacteria are members of the family Enterobacteriaceae and are defined as gram negative, non-spore-forming rods which ferment the sugar lactose with the evolution of gas and acids. Many coliforms are normally found in soil and water and do not necessarily indicate the presence of fecal contamination, but *Escherichia coli* (*E. coli*) is a primary bacterium in the human and animal intestinal tract and its presence in food or water indicates fecal contamination. Therefore, *E. coli* is the coliform that is used as an indicator for fecal contamination. Other coliform genera include *Citrobacter*, *Enterobacter* and *Klebsiella*. The USEPA acknowledges that *E. coli* is the best indicator of health risk in fresh water and is currently recommending testing for *E. coli* instead of fecal coliforms. The term "fecal coliform" indicates coliforms which will grow at a temperature of 44.5°C. This is not an accurate designation as there are coliforms of non-fecal origin that will grow at 44.5°C and there are strains of *E. coli* that will not grow at 44.5°C. Traditional tests for coliforms and *E. coli* or fecal coliforms require the inoculation of media containing lactose, incubation under carefully controlled temperatures, and examination for the presence of gas from lactose fermentation. Additional special media must then be inoculated and incubated at elevated, carefully controlled temperatures to confirm the presence of *E. coli* or fecal coliforms. All these require extra equipment and careful regulation of time and temperature. This approach is not only expensive and time consuming, but can be less than precise in indicating the numbers of specific organisms present.

As a result of the difficulties and lack of precision inherent in the older technology, new approaches have been developed and are being used very successfully. One of the best approaches is based on the fact that in order for coliforms to ferment lactose, they must produce certain enzymes which can be identified and used to verify the presence of the coliforms. General coliforms produce the enzyme galactosidase in lactose fermentation and *E. coli* produces the enzyme glucuronidase in addition to galactosidase. Coliscan takes advantage of these facts to give you a simple, accurate and quantitative way to identify and differentiate coliforms and *E. coli* (true fecal coliform) from other bacteria in water or other types of samples. This patented method incorporates two special chromogenic substrates which are acted upon by the presence of the enzymes galactosidase and glucuronidase to produce pigments of contrasting colors. All that is needed to identify the presence and numbers of coliforms and *E. coli* is to add a test sample to the medium, pour it into a petri dish and incubate it at room temperature or at a higher controlled temperature (35°C is suggested). General coliforms will produce the enzyme galactosidase and the colonies that grow in the medium will be a pink color. *E. coli* will produce both galactosidase and glucuronidase and will therefore grow as dark blue to purple colonies in the medium. It is simple to count the blue/purple colonies (*E. coli*) which indicate the number of *E. coli* per sample. The pink colonies indicate the number of general coliforms per sample. The combined general coliform and *E. coli* number equals the total coliform number. Any non-colored colonies which grow in the medium are not coliforms, but may be members of the family Enterobacteriaceae. Since the Coliscan contains inhibitors, most other bacterial types will not grow. It is best for the

Coliscan to be incubated at a temperature higher than room temperature so that the organisms will grow faster. The suggested temperature range is between 30-37°C (85-99° F). The coliform/E. *coli* organisms will grow faster at this temperature range than at room temperature, so that results can be counted at 24-48 hours incubation time instead of about 24 hours later if incubated at room temperature, 22-27°C (72-80°F). Micrology Laboratories can provide information on home made or inexpensive commercial incubators.

The beauty of the Coliscan method is that it uses proven and accepted technology to allow anyone to do effective coliform/E. *coli* testing. For water testing, you can add up to a 5 mL sample of water to the bottle of medium that makes one petri plate. This will detect as small a number of coliforms or *E. coli* as one living bacterium in five milliliters of water. The method is also easily adapted for large samples with membrane filter use. Beware of copycat methods by other manufacturers who claim similar red and blue colors for coliforms and fecal coliforms, but whose results are unreliable due to inferior technology. They cannot legally copy the patented Coliscan technology. Coliscan has a shelf life of 1 year and should be kept frozen until used. You may refrigerate for up to 2 weeks, but freezing is best in order to maintain color intensity throughout the 1 year period.

Appendix B

International Baccalaureate Extended Essay

By Gibbs Pearson, Fairhope High School

EE

One does not have to be able to analyze water, have a degree in biology, read the Alabama Water Watch booklet, watch the news, or even pick up a newspaper to know the fact that Fish River is a contaminated water source, for this is something well known by the residents of Baldwin County. For years the fish caught out of this river have been inedible due to this contamination, though most people associated the fish with high mercury levels which also remains a problem in Fish River. The severity of this contamination, unfortunately, is either unknown or not understood by the citizens of the area. While some people may believe the river to be slightly polluted, it is actually one of the ten most endangered places in the South (according to Southern Environmental Law Center). Fish River is included on the 303(d) list, a section of the Clean Water Act that requires states to list the waters that do not meet the preset water quality standards. This list includes contaminated waters from across the state and tells the reason for the water being on the list. On the list Fish River is said to be contaminated by both metals (mercury) and pathogens (E-coli), and also reports that the source of this contamination is unknown or possibly pasture grazing. The level of importance of this contamination is increased by the fact that Fist River is one of the two main tributaries to Weeks Bay, causing the pollution to expand into a larger body of water. Weeks Bay is just a small segment of Mobile Bay which also connects to the Gulf of Mexico, Both Mobile Bay and the Gulf of Mexico are on the 303(d) list with the causes being the same as Fish River, mercury and pathogens. There is no doubt that Fish River in some part is a cause of contamination of these two larger bodies of water.

When people Travel to Fish River pollution is not what comes to mind, for to the naked eye the river is reasonably clean with a very small amount of waste visible. This causes many residents to wonder, what is causing this river to be considered contaminated? The pollution of Fish River is not a visible thing, but is rather a pollution of the actual water in the form of bacteria, E-coli in particular. E-coli or Escherichia coli is a type of Bacteria Found in the wastes and Digestive Systems of most mammals. This type of bacteria can exist in over 700 different forms, and though the vast majority of these forms are completely harmless there are some forms that are highly pathogenic. A pathogenic Organism is one that causes diseases and these organisms happen to be abundant in Fish River. These harmful bacteria in this river cause damage to Humans, Fish, Birds, and Mammals, mostly anything that comes into contact with them. Swimming in the river brings a person's skin into direct contact with the pathogens which can cause skin problems, and swallowing the water can make one sick. These bacteria not only have the ability to make a person or animal slightly ill, they are able to cause crop disease, food poisoning, tooth decay, lock jaw and other various diseases. Often these bacteria are able affect seafood and the animals or people that consume this tainted food source.

The next question one should ask is where are these bacteria coming from? Like stated before the bacteria come into the environment through wastes, and this can be waste from most animals and every mammal. In the past studies have been done to prove that Human waste, cattle waste, and wildlife waste all contain E-coli. In Baldwin County and the Fish River watershed in particular there is an abundant supply of all three of these sources, each one being a possibility for the majority of the E-coli.

How does enough waste from any of these possible sources get into Fish River to cause it to be considered dangerous? Depending on the source there are different possibilities of how this bacterium enters the river. For the wildlife source, any amount of rain will carry the waste of wild animals into the river as runoff. Seeing as E-coli levels in the river are always high but are increased after a rain fall the possibility of this being the correct source increases. Fortunately the extremely high pathogen levels seem to be too great for the wild life to be the major cause, though it may be a minor one, and therefore it was not extensively studied as a threat of being a possible source. Cattle, similar to wildlife, enter the river through water runoff. This is a greater possibility that wildlife due to the abundance of cattle in the Fish River watershed. Most cattle reside near the river and some owners participate in pasture grazing strategies making the cattle a very possible source, and the source that "the powers that be" in the state department believe to be the cause of the pathogens in Fish River. Cattle grazing strategies can cause an increase in the amount of waste added to the river by cattle, and the most commonly used in the Fish River watershed is Rotational grazing, where cattle spend a specific amount of time in a closed off area before being moved to a different section of a field. This becomes a problem for the grass in that specific area becomes much less thick causing there to be a great difference in the filtration of water runoff as it passes through the vegetation. The increased pathogen numbers after rainfall also point to this being the source. The last possible source is humans and this also has a good chance of being major problem. Possibilities such as: failing septic systems, more sewer lines increasing the likelihood of leaks, waste water treatment plants discharging into Fish River, and the possibility of urban storm water runoff cause the human source option to be very probable as well. The man I worked on this project with is named Mike Shelton, who is the Watershed Coordinator at the Weeks Bay Fish and Wildlife Reserve, and after a career of working in the field of biology, in particular with fish and wildlife, his professional opinion of the situation is that the source is originating from Humans. He feels that there are not enough head of cattle in the watershed to cause the level of contamination seen in Fish River, especially sense the river has stopped being the primary water source for cattle and owners recently been required to fence off the river no longer allowing access for the cattle wither to drink or lower their body temperature. This exclusion of cattle directly from the river has caused owners to provide an alternative water source for the livestock, but has not greatly influenced the levels of pathogens in the river. The lack of a drastic change following the exclusion of cattle from the river leads Mr. Shelton to believe the source is elsewhere.

How does one go about determining which source is causing the majority of Fish River's pollution problem? This is where Mr. Shelton and I come in. The project I accomplished was not finding the source of E-coli in Fish River, but rather a subsection of the experiment. The project I undertook was to gather, dilute, and plate known sources of E-coli from the Fish River watershed. Once this section of the overall experiment was completed our results as well as water samples collected from different sections of the river at diverse water levels were to be sent to the University of West Alabama where, using a more extensive laboratory, they were to pinpoint the source of E-coli in Fish River. Once this project is completed the source of the pathogens plaguing Fish River will be exposed, therefore allowing actions to be made in order to control the exposure of E-coli to the surrounding environment of Fish River.

As stated earlier I assisted the Weeks Bay Watershed Coordinator, Mike Shelton, in gathering and plating known sources of E-coli. During this experiment we, Mr. Shelton and I, were assisted by representatives of local Waste Water Treatment Plants, and local owners of cattle. Our primary objective in this experiment was to successfully culture E-coli colonies on bacteria cultivating plates, and have a decent number of colonies from both human and cattle waste to send to the University of West Alabama for testing.

This experiment in completion took about a week of work, and was done at the Weeks Bay Reserve in the laboratory that is there. The experiment first began with me calling Mike Shelton, the Watershed Coordinator at the Weeks Bay Reserve, about the project and his accepting me as a coworker on the experiment. The first day consisted mostly of planning. We had to plan how to get waste from cows and people from different places within a day of each other, though it would be preferred to gather it all on the same day. We discussed possible areas to gather cow and human waste and how to go about gathering those. We then planned to meet in about three days and I went home with the task of contacting friends or relatives with livestock.

When I returned to the wildlife center I had one definite place to gather the cow waste, though we needed about two or three to ensure that a range of livestock waste from around the Fish River watershed was used in the experiment. This need of diversity helps to eliminate or mask possible malfunctions with the cells in the waste that could be due to a particular fertilizer or insecticide used around one specific heard of cattle. We then began to contact local Waste Water Treatment Plants for their permission to take some of their effluent. We were able to gather consent from Charlie Baumhauer, Public Relations at the Plantation Hills Waste Water Treatment Plant, Bobby Wood, the Superintendent at the city of Loxley's Waste Water Treatment Plant, and Dan McCrory, the superintendent at the city of Fairhope's Waste Water Treatment Plant. Both plants with a city in its name are owned by that respective city. Only the Plantation Hills plant is a privately owned plant and is situated at the northern end of Daphne. The reason these three Waste Water Treatment Plants were chosen because they all three reside within the Fish River watershed and they all three dump their "clean" product into the river. Once we attempted to find other sources of cattle fecal matter and were unsuccessful, the decision was made that we would have to resort to knocking on the doors of homes asking to have some of the waste from their livestock.

I returned four days later having collected the cow feces from the relation I had mentioned earlier that week as being an assured place to gather cattle manure from. Once I arrived we placed the manure I had already gathered and prepared to go collect the other samples we needed. We first went to the Plantation Hills Waste Water Treatment Plant to gather effluent. When we arrived we were shown to a place where we could use a dipper, a measuring cup attached to a rod, to easily scoop some of the Waste Water Treatment Plant's effluent, prior to being treated, and poor it into a plastic jar that we brought with us. While there I was taught how the waste water was treated at the plant using aeration treatment, or the adding of oxygen to the water causing the bacteria to become excited and destroy most of the waters contamination, and also using extremely powerful ultra violet lights to kill the bacteria before exiting the plant. This brief lecture on the inner working of a Waste Water Treatment Plant shows that what the plants purposefully put out into the environment is clear of harmful bacteria, and in turn saying that if the source is human it is either from septic tank failures or the sewage lines leaking before reaching

the plant. Once we gathered the effluent from the Plantation Hills Waste Water Treatment Plant the three human samples, including the samples from the Fairhope and Loxley Waste Water Treatment Plants that Mr. Shelton had gathered earlier that day, were enough to continue the experiment following the acquiring more cow manure. We then began working our way back south towards the wildlife center stopping and asking for cow waste on our way. We were able to gather two more samples from different pastures to add to the one I brought that morning, and with three cow samples and three human samples we returned to the lab to begin the next stage of the experiment. Once we returned to the laboratory we began the process of plating our samples which begins with dilutions. For the dilutions we used pre sterilized 1ml droppers, 100ml graduated cylinders, and distilled, sterilized water. We began with the human effluents and, using the graduated cylinder, measured out 50ml of each effluent, and I then combined them by placing all 150ml of effluent into a plastic container and vigorously shaking the mixture until the three mixtures were equally dispersed between each other. The reason for combining the three is to mix the different cells from different locations to help to avoid the possibility of gathering only the contaminated sample and then having to redo the entire process. After the mixture settled, using a sterile dropper and a graduated cylinder, I added 1ml of the effluents to 1000ml of water to create a ratio of 1:1000 effluents to water, and with the use of parafilm I was able to mix the dilution without taking it out of the graduated cylinder. With the standard dilution thoroughly mixed we began to create the dilutions to be plated, for the 1:1000 dilution could not be used because according to Mr. Shelton the colonies on the plate would be too numerous to count and would even be hard to separate from one another due to the over abundance of E-coli that would be present. To dilute the solution even farther we took 10ml of the 1:1000 standard mixture and added it to 100ml of distilled water to create a ratio of 1:10,000 effluent to water. The next dilution was to be a ratio of 1:100,000. I did this by taking 1ml of the standard dilution and then adding it to 100ml of water before mixing the contents thoroughly. For the final dilution, again 1ml was taken from the original dilution only this time it was added to 1000ml of water to create an effluent to water ratio of 1:1,000,000. Once these three dilutions were created we began this process with the cow waste. The cow manure dilutions began by taking approximately 1g of each of the samples and adding this to 3000ml of water to create the initial 1:1000 ratio of waste to water. Once this was thoroughly homogenized it was further diluted. By using 10ml of the original dilution and 100ml of water the 1:10,000 waste to water dilution was created. For the next two dilutions only 1ml each of the primary solution was needed. To create the 1:100,000 waste to water dilution the initial mixture was added to 100ml of water, and for the 1:1,000,000 waste to water dilution the original mixture was added to 1000ml of water. When we finally finished the diluting process the next step of plating the dilutions began. In order to be sure of our results we decided to plate each dilution twice, for this would allow us to see a drastic difference in the number of colonies for the same dilution and suspect contamination. To plate these dilutions and have them grow colonies they had to be first mixed with a media, and the media Mr. Shelton ordered was specially designed to culture coli bacteria. This media comes frozen in small bottles and includes sterile petri dishes. Once thawed I added 1ml of each dilution to a bottle and then mixed the new solutions, and I then repeated the process to have two of each different dilution of both human samples and cow samples. We also created a media mixed only with the distilled

water to use as a control and to check for contamination. Once mixed the newly made solutions were poured into petri dished and placed into the incubator to cultivate bacteria.

When I returned the next day to check the petri dishes the results were not up to expectations. The human dilutions showed very little E-coli on plates above the 1:10,000 dilutions, not enough to be counted and sent to the University of West Alabama for testing. Using the effluent that was made the day before, which had been kept chilled for the possibility of these kinds of results, Mr. Shelton and I created new waste to water dilutions of 1:5000 and 1:1000 to plate and reenter into the incubator. These less dilute mixtures were made to increase the number of E-coli colonies on the plates making them easier to isolate. The 1:10,000 human dilutions produced a good number of E-coli colonies and were placed into the refrigerator to slow the colonies growth until they could be counted and shipped with the other plates. The cow plates produced even less efficiently than the human plates, having little to no signs of E-coli colonies on the plates. In order to redo these plates new dilutions would need to be made but seeing as the cow manure was not chilled new samples had to be gathered.

Returning two days later I first examined the newly made human plates, which Mr. Shelton told me had produced well enough to be sent off. The first thing that we did was examine the human plates and count the E-coli colonies. The counting was fairly simple seeing that E-coli colonies appear a dark blue where as other bacteria appear red. The dilutions produced seventy five total colonies for six plates; the 1:10,000 plates produced eighteen colonies, the 1:5000 plates produced twenty-six colonies, and the 1:1000 plates produced thirty-one colonies. All of these plates were then placed in the refrigerator for the purpose of slowing the colony growth and to keep the plates fresh. The next step in the process was again gathering cow manure, which we did from the same locations as the first time. We began the second dilution process differently from the first, taking 2g of each waste sample and adding it to 200ml of water. Once properly mixed 1ml per plate was used to create three plates of 1:33 ratio of waste to water. Then 10ml of the 1:33 dilution was added to 100ml of water and then to 1000ml of water to make dilutions of 1:330 and 1:3300 respectively. These other dilutions were plated three times by taking 1ml amounts of the dilutions and adding them to the media, and then from the media bottles to the plates to be incubated.

When I returned the next day the results were still not as I had hoped for, but this time there were enough cells to be shipped off to be tested at the University of West Alabama. In total these plates produced 431 cell colonies even though the 1:3300 was the only one that was able to be used, for the other dilutions produced too many colonies to count.

Once Mr. Shelton and I had gathered enough E-coli colonies from the two major sources there were expressed shipped to the University of West Alabama for Multiple Antibiotic Resistance Testing. This testing is how the sources or E-coli in Fish River would be pinpointed, either to humans of cattle. This testing is an extensive process that uses natural antibiotic resistance, as well as medically formed resistances found in humans, to determine the source of pathogens in Fish River. This process works by creating patterns that coexist in both the Fish River water and either cattle of human waste. The process consists of taking colonies of E-coli from cattle and then exposing these colonies to multiple antibiotics, recording which antibiotics the E-coli resisted and which it didn’t. The next step of this process is to repeat the first step with the E-coli

colonies from human waste, again recording the results of different antibiotics. Depending on the number of water samples gained from Fish River, this process will be repeated for every water sample available. Once all of the samples have been exposed to numerous antibiotics the results will be compared. If the E-coli in Fish River is resistant to the same things as the human waste one can conclude that the source of E-coli in the river is humans. If the human waste does not match but the cattle manure does then the conclusion with be drawn that cattle are the source of E-coli in Fish River. If neither of these sources match, the experiment must begin a new looking for different possible sources in the river.

Upon determining that the source is either cattle of humans the process to control the escape of E-coli into the environment can begin. If the source turns out to be cattle the ability of owners to allow their livestock to graze freely would be terminated. The use of Fish River as a water source would be terminated and harsher laws preventing owners from allowing their cattle into the river would be passed. Most likely a law creating a distance that call cattle must be kept from the river would be introduced and management of pasture graving would come into effect. The purpose of these new regulations would be to keep cattle and in particular cattle waste as far from Fish River as possible. If the source proves to be from human waste different actions will be taken. The first action with be the testing of sewage lines for leaks and cracks in the piping. Many communal septic tanks would be checked for failure or leaks and a strong suggestion to private owners of septic tanks to have their tanks examined as well. If the source turns out to be neither of these and ends up being wildlife actions to prevent the E-coli produced by these animals entering Fish River will also be made.

Appendix C

Origin, discriminant function scores, classification, probability, and distances from the group center of all *E. coli* isolates

Discriminant Function Analysis

Sample	Actual Group	Predicted Group (**misclassification)	Group Statistics		Discriminant Scores	
			Probability	Distance to group center	Function 1	Function 2
1	2	2	.999	.001	-.008	-.389
2	1	3**	.957	.087	.706	.446
3	1	3**	.939	.127	.539	.146
4	1	3**	.870	.280	1.405	.326
5	1	3**	.828	.377	.291	.353
6	1	3**	.376	1.956	-.156	1.144
7	1	2**	.996	.009	.055	-.286
8	1	2**	.941	.122	-.282	-.568
9	1	2**	.900	.211	-.268	.019
10	1	2**	.853	.318	.476	-.061
11	1	2**	.805	.435	.392	.171
12	1	2**	.748	.580	-.472	.244
13	1	2**	.662	.826	-.519	.392
14	1	2**	.613	.978	-.878	-.818
15	1	2**	.577	1.100	-.254	-1.375
16	1	2**	.531	1.265	-1.090	-.651
17	1	2**	.435	1.665	.722	-1.424
18	1	1	.933	.138	-1.067	-.155
19	1	1	.876	.266	-1.022	-.299
20	1	1	.861	.298	-1.317	-.258
21	1	1	.824	.386	-1.531	.605
22	1	1	.726	.639	-1.292	.977
23	1	1	.655	.846	-1.916	.515
24	1	1	.476	1.483	-2.243	.440
25	1	1	.416	1.753	-2.262	.740
26	1	1	.416	1.753	-2.262	.740
27	1	1	.400	1.833	-2.012	-.732
28	1	1	.391	1.877	-2.120	1.068
29	1	1	.371	1.981	-2.294	.868
30	1	1	.364	2.019	-1.846	1.391
31	1	1	.360	2.041	-2.403	.665
32	1	1	.349	2.105	-1.979	-.895
33	1	1	.313	2.326	-2.400	.918
34	1	1	.304	2.380	-2.543	.590
35	1	1	.304	2.380	-2.526	.653
36	1	1	.300	2.406	-2.492	.778
37	1	1	.253	2.749	-2.649	.641
38	1	1	.201	3.209	-2.823	.440
39	1	1	.201	3.209	-2.823	.440
40	1	3**	.934	.137	.536	.324
41	1	3**	.919	.168	1.114	.556
42	1	2**	.845	.336	-.298	.143
43	1	2**	.832	.367	-.570	-.142
44	1	2**	.821	.395	.514	-.001

45	1	2**	.769	.524	-.447	.216
46	1	2**	.693	.735	-.565	.291
47	1	2**	.542	1.226	-.273	.718
48	1	2**	.430	1.690	-1.060	-1.115
49	1	2**	.279	2.553	-.972	-1.628
50	1	1	.994	.012	-.939	.234
51	1	1	.992	.016	-.968	.314
52	1	1	.962	.077	-.853	.417
53	1	1	.949	.106	-1.211	-.064
54	1	1	.947	.108	-1.346	.352
55	1	1	.926	.153	-.976	.601
56	1	1	.695	.727	-1.850	.499
57	1	1	.610	.987	-1.424	1.135
58	1	1	.388	1.895	-2.114	1.085
59	1	1	.329	2.226	-1.903	-1.005
60	1	1	.192	3.298	-1.949	1.792
61	2	3**	.628	.932	.997	-.746
62	2	3**	.460	1.554	1.794	-.642
63	2	3**	.323	2.258	1.741	-1.024
64	2	3**	.000	26.402	3.146	4.829
65	2	1**	.779	.500	-1.646	-.158
66	2	1**	.764	.539	-1.245	-.490
67	2	1**	.569	1.128	-2.064	-.085
68	2	1**	.381	1.932	-.632	1.543
69	2	1**	.224	2.990	-.339	1.794
70	2	2	.954	.094	.176	-.108
71	2	2	.944	.114	-.152	-.053
72	2	2	.919	.170	-.287	-.656
73	2	2	.904	.202	-.088	-.798
74	2	2	.883	.250	.483	-.245
75	2	2	.856	.311	-.561	-.375
76	2	2	.806	.432	-.659	-.403
77	2	2	.798	.452	-.197	-1.001
78	2	2	.753	.568	-.444	-.968
79	2	2	.744	.592	-.360	-1.039
80	2	2	.741	.599	-.585	-.868
81	2	2	.727	.637	-.167	-1.138
82	2	2	.700	.712	-.484	-1.051
83	2	2	.661	.828	.000	-1.266
84	2	2	.647	.871	-.054	-1.288
85	2	2	.549	1.200	-.464	-1.350
86	2	2	.549	1.200	.397	-1.376
87	2	2	.531	1.265	-.788	-1.163
88	2	2	.518	1.316	-.540	-1.371
89	2	2	.484	1.450	-.082	-1.558
90	2	2	.475	1.488	-.663	-1.383
91	2	2	.470	1.511	.865	-1.226
92	2	2	.466	1.527	.813	-1.283
93	2	2	.412	1.773	-.561	-1.566

94	2	2	.408	1.791	1.042	-1.191
95	2	2	.405	1.809	-.768	-1.463
96	2	2	.390	1.883	.008	-1.728
97	2	2	.383	1.920	.901	-1.406
98	2	2	.249	2.778	-.801	-1.820
99	2	2	.230	2.936	.064	-2.069
100	2	2	.139	3.951	-.063	-2.343
101	2	2	.021	7.739	-.415	-3.108
102	2	2	.005	10.679	.087	-3.623
103	2	2	.001	13.886	.672	-4.021
104	2	3**	.986	.027	.851	.053
105	2	3**	.966	.070	.910	-.050
106	2	3**	.843	.341	1.249	.674
107	2	3**	.777	.504	.318	.636
108	2	3**	.550	1.197	1.968	.034
109	2	3**	.521	1.304	.480	1.280
110	2	3**	.407	1.797	1.255	1.503
111	2	3**	.314	2.317	2.408	.305
112	2	3**	.196	3.257	1.376	1.951
113	2	1**	.990	.021	-.991	.351
114	2	1**	.989	.023	-1.128	.342
115	2	1**	.943	.117	-.723	.329
116	2	1**	.871	.275	-1.024	-.308
117	2	1**	.765	.537	-.740	.882
118	2	1**	.727	.638	-1.703	.671
119	2	1**	.628	.931	-.455	.980
120	2	1**	.597	1.033	-.960	1.229
121	2	1**	.515	1.327	-.224	1.024
122	2	2	.999	.001	-.008	-.389
123	2	2	.982	.036	-.189	-.314
124	2	2	.952	.098	-.202	-.114
125	2	2	.900	.211	-.268	.019
126	2	2	.890	.233	-.217	.076
127	2	2	.889	.235	.467	-.469
128	2	2	.854	.316	-.265	.142
129	2	2	.840	.349	.184	-.917
130	2	2	.809	.424	.116	.284
131	2	2	.801	.443	-.031	.309
132	2	2	.778	.503	.179	.329
133	2	2	.750	.576	-.180	.382
134	2	2	.745	.587	-.124	.400
135	2	2	.742	.596	-.388	.313
136	2	2	.710	.684	.147	.457
137	2	2	.643	.882	-.345	.519
138	2	2	.628	.931	-.118	.602
139	3	2**	.874	.270	.483	-.174
140	3	2**	.841	.346	.584	-.357
141	3	2**	.777	.504	-.127	-1.056
142	3	2**	.738	.609	.163	.406

143	3	2**	.692	.738	.821	-.597
144	3	2**	.622	.949	.673	-1.057
145	3	2**	.595	1.037	.771	-1.017
146	3	2**	.558	1.167	.197	-1.418
147	3	2**	.511	1.342	-.941	-1.038
148	3	2**	.439	1.648	.602	-1.488
149	3	2**	.349	2.105	.646	-1.654
150	3	2**	.047	6.120	1.617	-2.225
151	3	1**	.726	.639	-1.836	.093
152	3	1**	.525	1.290	-.903	1.343
153	3	1**	.438	1.652	-.258	1.232
154	3	1**	.328	2.228	-1.117	1.707
155	3	3	.940	.125	1.136	.465
156	3	3	.921	.164	.496	.111
157	3	3	.914	.181	.979	-.202
158	3	3	.781	.494	1.591	.199
159	3	3	.772	.517	1.606	.253
160	3	3	.739	.606	1.441	.762
161	3	3	.738	.607	.979	.988
162	3	3	.689	.745	1.699	-.083
163	3	3	.678	.778	1.685	.593
164	3	3	.615	.972	1.820	.535
165	3	3	.590	1.054	.950	1.239
166	3	3	.572	1.116	1.919	.445
167	3	3	.559	1.163	1.695	.930
168	3	3	.558	1.166	1.784	.817
169	3	3	.507	1.358	2.043	.055
170	3	3	.407	1.796	.027	1.240
171	3	3	.257	2.720	2.516	-.053
172	3	3	.185	3.371	2.156	-1.114
173	3	3	.067	5.408	2.517	-1.447
174	3	3	.030	7.020	3.500	-.232
175	3	3	.011	9.064	3.874	-.174
176	3	2**	.993	.013	.087	-.427
177	3	2**	.909	.191	.163	.048
178	3	2**	.907	.195	.437	-.332
179	3	2**	.874	.268	.384	-.700
180	3	2**	.867	.285	.035	.176
181	3	2**	.848	.330	-.023	.218
182	3	2**	.828	.376	-.171	.234
183	3	2**	.807	.428	.239	.251
184	3	2**	.660	.833	-.102	.551
185	3	1**	.730	.629	-.592	.866
186	3	3	.985	.030	.759	.329
187	3	3	.976	.049	.707	.087
188	3	3	.974	.052	.881	-.014
189	3	3	.969	.063	.638	.237
190	3	3	.968	.066	.640	.278
191	3	3	.965	.070	1.137	.120

192	3	3	.947	.109	1.105	.462
193	3	3	.928	.148	.985	.587
194	3	3	.908	.193	.661	.590
195	3	3	.892	.230	.615	.607
196	3	3	.888	.238	.441	.407
197	3	3	.827	.380	.296	.384
198	3	3	.795	.458	.966	.886
199	3	3	.780	.497	1.352	.744
200	3	3	.741	.601	.467	.864
201	3	3	.701	.712	.664	1.027
202	3	3	.644	.880	.159	.804
203	3	3	.566	1.138	1.953	.283
204	3	3	.525	1.290	.613	1.315
205	3	3	.414	1.766	.254	1.381
206	3	3	.382	1.922	2.252	-.038
207	3	3	.362	2.035	1.948	1.169
208	3	3	.213	3.091	2.374	1.154
209	3	3	.007	9.979	2.963	2.596
210	ungrouped	3	.867	.284	.538	.616
211	ungrouped	3	.974	.053	.664	.266
212	ungrouped	3	.961	.080	.964	-.059
213	ungrouped	3	.806	.432	.485	.733
214	ungrouped	3	.723	.648	1.162	-.543
215	ungrouped	3	.520	1.309	.769	1.352
216	ungrouped	2	.500	1.385	.140	-1.525
217	ungrouped	3	.982	.036	.727	.311
218	ungrouped	3	.889	.236	.709	-.237
219	ungrouped	3	.864	.293	.444	.523
220	ungrouped	3	.815	.409	.789	.846
221	ungrouped	3	.741	.600	.912	.988
222	ungrouped	3	.643	.882	1.755	.576
223	ungrouped	3	.319	2.286	.045	1.469
224	ungrouped	2	.980	.040	-.198	-.311
225	ungrouped	2	.950	.102	-.100	-.662
226	ungrouped	2	.929	.147	.362	-.241
227	ungrouped	2	.823	.389	.209	.230
228	ungrouped	2	.552	1.190	-.383	-1.379
229	ungrouped	2	.205	3.166	.983	-1.837
230	ungrouped	1	.964	.073	-.921	-.024
231	ungrouped	2	.754	.565	-.524	.187
232	ungrouped	3	.968	.066	1.004	-.015
233	ungrouped	3	.941	.121	1.228	.291
234	ungrouped	3	.897	.216	1.105	-.198
235	ungrouped	3	.875	.267	1.067	-.271
236	ungrouped	3	.833	.366	1.214	-.296
237	ungrouped	3	.778	.502	1.493	-.156
238	ungrouped	3	.721	.653	1.358	-.444
239	ungrouped	3	.718	.662	1.315	-.479
240	ungrouped	2	.952	.099	.220	-.135

241	ungrouped	2	.948	.106	-.087	-.672
242	ungrouped	3	.910	.188	.458	.168
243	ungrouped	3	.812	.415	1.038	.841
244	ungrouped	3	.807	.428	1.349	.678
245	ungrouped	3	.797	.453	.504	.767
246	ungrouped	3	.757	.557	.534	.871
247	ungrouped	2	.879	.258	.217	.101
248	ungrouped	2	.751	.573	-.397	.290
249	ungrouped	2	.742	.597	-.639	.084
250	ungrouped	2	.736	.614	-.247	.388
251	ungrouped	2	.722	.651	-.148	.438
252	ungrouped	1	.920	.167	-.734	.481
253	ungrouped	1	.773	.515	-.504	.687
254	ungrouped	1	.751	.572	-.599	.826
255	ungrouped	3	.957	.087	.788	.491
256	ungrouped	3	.950	.103	1.153	.396
257	ungrouped	3	.863	.295	.719	.730
258	ungrouped	3	.842	.344	.360	.468
259	ungrouped	3	.818	.401	.302	.453
260	ungrouped	3	.535	1.250	.279	1.151
261	ungrouped	3	.065	5.476	2.678	1.722
262	ungrouped	2	.990	.019	.025	-.220
263	ungrouped	2	.990	.020	-.062	-.229
264	ungrouped	2	.976	.048	-.215	-.418
265	ungrouped	2	.961	.080	.237	-.506
266	ungrouped	2	.913	.182	-.012	-.783
267	ungrouped	2	.894	.223	-.029	.115
268	ungrouped	2	.878	.260	.494	-.245
269	ungrouped	2	.850	.326	.283	.137
270	ungrouped	2	.842	.344	.583	-.359
271	ungrouped	2	.822	.392	.537	-.040
272	ungrouped	2	.819	.400	-.628	-.257
273	ungrouped	2	.797	.454	.124	.305
274	ungrouped	2	.757	.558	.084	-1.098
275	ungrouped	2	.729	.633	.092	.433
276	ungrouped	2	.686	.753	-.232	.481
277	ungrouped	2	.653	.852	-.573	-1.083
278	ungrouped	2	.535	1.251	1.012	-.825
279	ungrouped	2	.321	2.272	-1.241	-1.217
280	ungrouped	2	.180	3.429	-1.420	-1.549
281	ungrouped	3	.988	.025	.762	.306
282	ungrouped	3	.732	.625	.275	.713
283	ungrouped	3	.498	1.393	2.009	.583
284	ungrouped	2	.913	.182	-.316	-.065
285	ungrouped	2	.808	.427	.646	-.283
286	ungrouped	2	.783	.489	.307	.270
287	ungrouped	2	.690	.742	.705	-.846
288	ungrouped	2	.602	1.014	-.439	.552
289	ungrouped	2	.587	1.067	-.112	.671

290	ungrouped	3	.959	.084	.604	.267
291	ungrouped	3	.911	.186	.919	-.217
292	ungrouped	3	.806	.432	.485	.733
293	ungrouped	3	.703	.705	1.604	.653
294	ungrouped	3	.409	1.789	-.015	1.200
295	ungrouped	2	.987	.025	-.148	-.425
296	ungrouped	2	.938	.128	.351	-.403
297	ungrouped	2	.916	.175	-.252	-.020
298	ungrouped	2	.877	.263	-.449	-.100
299	ungrouped	2	.867	.286	-.099	.170
300	ungrouped	2	.856	.312	.111	.190
301	ungrouped	2	.791	.468	-.196	.300
302	ungrouped	2	.767	.531	.009	.372
303	ungrouped	2	.762	.543	-.500	.188
304	ungrouped	2	.719	.659	-.249	.418
305	ungrouped	2	.672	.795	.020	.535
306	ungrouped	2	.641	.889	.631	-1.053
307	ungrouped	2	.633	.916	-.029	.600
308	ungrouped	2	.560	1.160	-.081	.718
309	ungrouped	2	.464	1.534	-.071	.880
310	ungrouped	2	.455	1.573	-.428	-1.537
311	ungrouped	1	.895	.221	-.602	.370
312	ungrouped	1	.886	.242	-.596	.414
313	ungrouped	3	.966	.069	.827	-.043
314	ungrouped	3	.911	.186	.478	.348
315	ungrouped	3	.782	.492	.643	.871
316	ungrouped	3	.742	.596	.947	.983
317	ungrouped	3	.661	.827	.380	.968
318	ungrouped	3	.529	1.275	1.306	-.835
319	ungrouped	3	.390	1.885	.108	1.343
320	ungrouped	2	.990	.019	.078	-.469
321	ungrouped	2	.894	.225	.314	-.005
322	ungrouped	2	.890	.232	-.463	-.211
323	ungrouped	2	.881	.253	.125	.130
324	ungrouped	2	.869	.280	.518	-.274
325	ungrouped	2	.840	.349	-.309	.149
326	ungrouped	2	.826	.383	-.582	-.576
327	ungrouped	2	.809	.423	.510	-.755
328	ungrouped	2	.760	.548	-.460	.226
329	ungrouped	2	.734	.619	-.561	-.912
330	ungrouped	2	.705	.699	.206	.453
331	ungrouped	2	.602	1.014	-.439	.552
332	ungrouped	2	.435	1.665	-.198	-1.632
333	ungrouped	2	.347	2.115	-.278	-1.785
334	ungrouped	1	.993	.014	-1.024	.333
335	ungrouped	1	.936	.133	-.700	.330
336	ungrouped	1	.731	.626	-.438	.722
337	ungrouped	1	.667	.809	-.379	.820
338	ungrouped	2	.987	.025	.061	-.212

339	ungrouped	2	.987	.025	.061	-.212
340	ungrouped	3	.852	.321	.356	.408
341	ungrouped	3	.828	.377	.524	.708
342	ungrouped	3	.818	.403	.575	.766
343	ungrouped	3	.302	2.393	.260	1.627
344	ungrouped	2	.985	.029	-.136	-.247
345	ungrouped	2	.863	.295	.037	.185
346	ungrouped	2	.852	.319	-.308	.120
347	ungrouped	2	.650	.861	.044	.570
348	ungrouped	1	.989	.022	-1.117	.085
349	ungrouped	1	.962	.078	-.816	.376
350	ungrouped	1	.904	.202	-1.384	-.080
351	ungrouped	1	.718	.661	-1.665	.744
352	ungrouped	1	.549	1.198	-2.129	.058
353	ungrouped	3	.999	.002	.851	.235
354	ungrouped	3	.999	.002	.851	.235
355	ungrouped	3	.938	.128	.531	.231
356	ungrouped	3	.934	.136	.544	.346
357	ungrouped	3	.916	.174	.880	.631
358	ungrouped	3	.828	.378	.967	.824
359	ungrouped	3	.617	.966	.062	.745
360	ungrouped	3	.573	1.115	1.135	-.813
361	ungrouped	3	.550	1.196	1.115	1.284
362	ungrouped	3	.065	5.476	2.678	1.722
363	ungrouped	3	.065	5.476	2.678	1.722
364	ungrouped	3	.065	5.476	2.678	1.722
365	ungrouped	3	.065	5.476	2.678	1.722
366	ungrouped	3	.065	5.476	2.678	1.722
367	ungrouped	3	.065	5.476	2.678	1.722
368	ungrouped	3	.065	5.476	2.678	1.722
369	ungrouped	2	.979	.043	.174	-.250
370	ungrouped	2	.956	.091	.117	-.080
371	ungrouped	2	.945	.113	.252	-.138
372	ungrouped	2	.939	.125	.346	-.303
373	ungrouped	2	.938	.129	.353	-.320
374	ungrouped	2	.931	.142	-.355	-.493
375	ungrouped	2	.929	.147	.155	-.007
376	ungrouped	2	.880	.256	.251	.081
377	ungrouped	2	.874	.268	.482	-.176
378	ungrouped	2	.874	.269	.295	.068
379	ungrouped	2	.871	.276	.181	.136
380	ungrouped	2	.868	.283	-.526	-.256
381	ungrouped	2	.866	.287	.495	-.551
382	ungrouped	2	.855	.314	.291	.120
383	ungrouped	2	.852	.320	.353	.083
384	ungrouped	2	.831	.371	.107	.242
385	ungrouped	2	.772	.518	.232	.324
386	ungrouped	2	.761	.547	-.258	.338
387	ungrouped	2	.743	.594	.657	-.753

388	ungrouped	2	.699	.717	-.372	.406
389	ungrouped	2	.642	.886	.649	-1.035
390	ungrouped	2	.551	1.192	.846	-1.043
391	ungrouped	2	.539	1.238	.119	-1.462
392	ungrouped	2	.491	1.421	-.961	-1.067
393	ungrouped	2	.025	7.409	-.610	-3.010
394	ungrouped	1	.835	.360	-1.415	.690
395	ungrouped	1	.952	.098	-1.160	-.075
396	ungrouped	3	.338	2.168	1.378	1.602
397	ungrouped	2	.921	.165	-.392	-.238
398	ungrouped	2	.833	.366	-.603	-.442
399	ungrouped	2	.769	.526	-.384	.261
400	ungrouped	2	.653	.852	-.419	-1.180
401	ungrouped	2	.612	.981	-.612	-1.138
402	ungrouped	2	.478	1.475	-.897	-1.179
403	ungrouped	2	.441	1.637	-.891	-1.279
404	ungrouped	2	.434	1.669	-.986	-1.196
405	ungrouped	3	.653	.852	1.739	.571
406	ungrouped	3	.621	.952	.134	.832
407	ungrouped	2	.453	1.582	-.195	.887
408	ungrouped	2	.868	.284	.366	.027
409	ungrouped	2	.798	.452	.667	-.310
410	ungrouped	3	.985	.030	.729	.282
411	ungrouped	3	.919	.169	.972	-.189
412	ungrouped	3	.857	.308	.892	-.341
413	ungrouped	3	.790	.470	.261	.491
414	ungrouped	3	.740	.603	1.290	-.451
415	ungrouped	3	.708	.690	.988	-.611
416	ungrouped	2	.971	.059	.207	-.237
417	ungrouped	2	.933	.138	-.372	-.410
418	ungrouped	2	.912	.184	.424	-.316
419	ungrouped	2	.867	.286	.284	.094
420	ungrouped	2	.790	.472	.343	.236
421	ungrouped	2	.765	.537	.678	-.624
422	ungrouped	2	.732	.625	.122	-1.137
423	ungrouped	2	.713	.676	-.754	-.019
424	ungrouped	2	.696	.726	.773	-.706
425	ungrouped	2	.596	1.033	-.414	.574
426	ungrouped	2	.541	1.228	-.947	-.938
427	ungrouped	1	.911	.185	-1.473	.274
428	ungrouped	1	.911	.185	-1.473	.274
429	ungrouped	1	.898	.215	-.600	.341
430	ungrouped	1	.750	.575	-1.676	.640
431	ungrouped	3	.995	.010	.950	.290
432	ungrouped	3	.653	.854	.153	.773
433	ungrouped	3	.581	1.086	.352	1.107
434	ungrouped	2	.842	.343	-.275	.163
435	ungrouped	2	.832	.369	-.559	-.110
436	ungrouped	2	.805	.433	.185	.274

437	ungrouped	2	.760	.548	-.166	.366
438	ungrouped	2	.754	.566	-.696	-.062
439	ungrouped	2	.737	.609	-.748	-.119
440	ungrouped	2	.729	.632	-.376	.346
441	ungrouped	2	.668	.806	-.033	.541
442	ungrouped	2	.540	1.233	-.057	.753
443	ungrouped	1	.985	.030	-.970	.060
444	ungrouped	1	.900	.211	-1.001	.674
445	ungrouped	1	.845	.336	-.722	.697
446	ungrouped	1	.841	.346	-.967	.799
447	ungrouped	1	.489	1.431	-.180	1.042
448	ungrouped	3	.891	.232	.739	.671
449	ungrouped	3	.622	.950	1.162	1.149
450	ungrouped	3	.571	1.122	.013	.810
451	ungrouped	3	.225	2.987	.131	1.767
452	ungrouped	3	.195	3.270	.082	1.832
453	ungrouped	3	.159	3.679	.058	1.943
454	ungrouped	2	.945	.112	.319	-.446
455	ungrouped	2	.922	.162	-.125	.027
456	ungrouped	2	.914	.180	-.348	-.108
457	ungrouped	2	.823	.391	-.555	-.652
458	ungrouped	2	.817	.404	-.634	-.275
459	ungrouped	2	.817	.404	.316	.193
460	ungrouped	2	.735	.615	-.695	-.727
461	ungrouped	2	.734	.620	-.204	.405
462	ungrouped	2	.725	.643	-.431	.322
463	ungrouped	2	.496	1.402	-1.134	-.709
464	ungrouped	2	.402	1.821	-1.101	-1.142
465	ungrouped	2	.321	2.272	-1.241	-1.217
466	ungrouped	1	.970	.060	-1.288	.178
467	ungrouped	1	.955	.092	-1.022	.519
468	ungrouped	1	.945	.113	-1.211	.509
469	ungrouped	1	.920	.166	-1.327	-.079
470	ungrouped	1	.862	.297	-.550	.442
471	ungrouped	1	.727	.637	-.825	.983
472	ungrouped	1	.522	1.299	-.605	1.267
473	ungrouped	1	.440	1.641	-.677	1.443
474	ungrouped	3	.986	.029	.943	.375
475	ungrouped	3	.972	.057	1.076	.065
476	ungrouped	3	.950	.102	.592	.096
477	ungrouped	3	.948	.106	.722	.494
478	ungrouped	3	.946	.110	.680	.472
479	ungrouped	3	.878	.260	1.020	.706
480	ungrouped	3	.870	.277	.426	.465
481	ungrouped	3	.856	.310	.422	.517
482	ungrouped	3	.726	.641	1.146	.972
483	ungrouped	3	.644	.880	.624	1.114
484	ungrouped	3	.627	.932	1.458	.993
485	ungrouped	3	.484	1.453	1.595	1.190

486	ungrouped	3	.464	1.537	.311	1.311
487	ungrouped	3	.436	1.660	1.901	1.010
488	ungrouped	3	.391	1.879	1.891	1.149
489	ungrouped	3	.355	2.069	.077	1.401
490	ungrouped	3	.325	2.247	.654	1.694
491	ungrouped	3	.065	5.476	2.678	1.722
492	ungrouped	3	.065	5.476	2.678	1.722
493	ungrouped	3	.065	5.476	2.678	1.722
494	ungrouped	3	.065	5.476	2.678	1.722
495	ungrouped	3	.065	5.476	2.678	1.722
496	ungrouped	3	.065	5.476	2.678	1.722
497	ungrouped	3	.065	5.476	2.678	1.722
498	ungrouped	2	.987	.026	.002	-.197
499	ungrouped	2	.985	.030	.027	-.526
500	ungrouped	2	.981	.038	-.143	-.219
501	ungrouped	2	.946	.110	-.146	-.657
502	ungrouped	2	.929	.147	-.387	-.365
503	ungrouped	2	.928	.150	.370	-.257
504	ungrouped	2	.923	.160	-.244	-.037
505	ungrouped	2	.902	.207	-.456	-.310
506	ungrouped	2	.878	.260	-.450	-.109
507	ungrouped	2	.840	.348	-.139	.218
508	ungrouped	2	.828	.379	.594	-.209
509	ungrouped	2	.820	.396	.625	-.337
510	ungrouped	2	.816	.407	-.271	.223
511	ungrouped	2	.794	.462	.080	.318
512	ungrouped	2	.657	.839	-.091	.556
513	ungrouped	2	.553	1.184	-.338	-1.392
514	ungrouped	2	.509	1.352	-.024	.806
515	ungrouped	2	.489	1.430	1.009	-.992
516	ungrouped	1	.998	.005	-1.003	.161
517	ungrouped	3	.988	.023	.741	.251
518	ungrouped	3	.985	.031	.712	.200
519	ungrouped	3	.947	.109	.768	.520
520	ungrouped	3	.935	.135	1.090	.521
521	ungrouped	3	.926	.153	.506	.128
522	ungrouped	3	.912	.185	.783	.631
523	ungrouped	3	.904	.202	.891	.663
524	ungrouped	3	.900	.210	1.188	.561
525	ungrouped	3	.809	.424	.755	.851
526	ungrouped	3	.682	.765	1.503	.836
527	ungrouped	3	.679	.774	1.073	1.074
528	ungrouped	2	.965	.071	-.092	-.105
529	ungrouped	2	.871	.276	-.336	.050
530	ungrouped	2	.700	.714	-.584	.258
531	ungrouped	1	.840	.350	-.510	.466
532	ungrouped	3	.962	.078	1.167	.236
533	ungrouped	3	.903	.204	.587	.550
534	ungrouped	3	.814	.411	.455	.686

535	ungrouped	3	.732	.625	.275	.713
536	ungrouped	3	.590	1.055	.170	.948
537	ungrouped	2	.992	.017	-.004	-.228
538	ungrouped	2	.949	.104	.295	-.234
539	ungrouped	2	.945	.113	-.204	-.085
540	ungrouped	2	.909	.190	-.369	-.119
541	ungrouped	2	.866	.287	-.399	.005
542	ungrouped	2	.769	.524	.215	-1.047
543	ungrouped	2	.768	.529	-.718	-.218
544	ungrouped	2	.763	.542	.268	.327
545	ungrouped	2	.729	.632	-.039	.438
546	ungrouped	2	.722	.652	-.089	-1.159
547	ungrouped	2	.716	.669	-.812	-.481
548	ungrouped	2	.689	.746	-.088	.503
549	ungrouped	2	.625	.939	.897	-.713
550	ungrouped	2	.611	.987	.582	-1.159
551	ungrouped	2	.505	1.365	.106	-1.520
552	ungrouped	2	.413	1.769	.903	-1.329
553	ungrouped	2	.409	1.786	.511	-1.590
554	ungrouped	1	.994	.012	-.955	.152
555	ungrouped	1	.953	.096	-.855	.459
556	ungrouped	1	.081	5.033	-.834	2.450
557	ungrouped	3	.341	2.154	2.091	-.627
558	ungrouped	3	.994	.013	.795	.149
559	ungrouped	3	.806	.431	1.022	-.429
560	ungrouped	2	.964	.073	-.156	-.132
561	ungrouped	2	.964	.073	-.156	-.132
562	ungrouped	2	.941	.121	-.335	-.464
563	ungrouped	2	.903	.204	-.391	-.124
564	ungrouped	2	.902	.206	.316	-.034
565	ungrouped	2	.811	.420	.608	-.569
566	ungrouped	2	.775	.510	-.499	.158
567	ungrouped	2	.711	.683	.797	-.560
568	ungrouped	2	.585	1.072	-.389	-1.318
569	ungrouped	2	.538	1.238	-1.066	-.688
570	ungrouped	2	.317	2.296	-.184	-1.861
571	ungrouped	1	.942	.120	-.709	.138
572	ungrouped	1	.823	.389	-1.557	-.141
573	ungrouped	1	.521	1.304	-1.571	-.798
574	ungrouped	3	.924	.159	.614	.503
575	ungrouped	3	.865	.291	.396	.433
576	ungrouped	3	.801	.444	.675	.845
577	ungrouped	3	.737	.610	.665	.962
578	ungrouped	3	.702	.709	.169	.652
579	ungrouped	3	.687	.751	.681	1.055
580	ungrouped	3	.525	1.288	-.004	.915
581	ungrouped	2	.910	.188	-.332	-.074
582	ungrouped	2	.889	.235	.005	.129
583	ungrouped	2	.872	.275	-.081	.162

584	ungrouped	2	.821	.394	.323	.179
585	ungrouped	2	.819	.400	-.156	.257
586	ungrouped	2	.809	.423	.081	.289
587	ungrouped	2	.790	.472	-.241	.288
588	ungrouped	2	.693	.733	-.077	.497
589	ungrouped	2	.693	.735	-.269	.459
590	ungrouped	2	.658	.836	-.141	.547
591	ungrouped	2	.536	1.246	-.362	.701
592	ungrouped	1	.966	.069	-.784	.236
593	ungrouped	1	.757	.557	-.432	.641
594	ungrouped	1	.744	.592	-1.075	-.553
595	ungrouped	3	.994	.011	.786	.238
596	ungrouped	3	.976	.049	.870	-.008
597	ungrouped	3	.968	.064	1.105	.346
598	ungrouped	3	.962	.077	.677	.035
599	ungrouped	3	.959	.084	.882	-.077
600	ungrouped	3	.938	.128	.531	.231
601	ungrouped	3	.915	.177	.793	.623
602	ungrouped	3	.896	.220	.491	.463
603	ungrouped	3	.862	.297	.443	.528
604	ungrouped	3	.776	.508	1.313	.787
605	ungrouped	3	.747	.585	.188	.521
606	ungrouped	3	.685	.756	.227	.779
607	ungrouped	3	.644	.880	.682	1.129
608	ungrouped	3	.490	1.425	2.081	.266
609	ungrouped	3	.065	5.476	2.678	1.722
610	ungrouped	3	.065	5.476	2.678	1.722
611	ungrouped	2	.993	.014	.065	-.453
612	ungrouped	2	.993	.014	.065	-.453
613	ungrouped	2	.967	.068	-.176	-.161
614	ungrouped	2	.956	.091	-.044	-.655
615	ungrouped	2	.922	.163	-.067	.042
616	ungrouped	2	.915	.178	.282	-.047
617	ungrouped	2	.911	.186	-.418	-.237
618	ungrouped	2	.900	.211	.015	.102
619	ungrouped	2	.834	.363	.568	-.168
620	ungrouped	2	.827	.380	.089	-.966
621	ungrouped	2	.786	.482	-.660	-.129
622	ungrouped	3	.878	.260	.387	.309
623	ungrouped	3	.873	.272	.372	.286
624	ungrouped	1	.957	.089	-1.083	.512
625	ungrouped	1	.856	.310	-.702	.654
626	ungrouped	3	.995	.010	.977	.258
627	ungrouped	3	.988	.025	.735	.182
628	ungrouped	3	.981	.038	.753	.356
629	ungrouped	3	.980	.040	.761	.367
630	ungrouped	3	.952	.097	.966	-.089
631	ungrouped	3	.947	.108	.630	.416
632	ungrouped	3	.940	.123	1.152	.445

633	ungrouped	3	.905	.200	.442	.232
634	ungrouped	3	.843	.341	1.322	-.177
635	ungrouped	3	.834	.364	1.355	.595
636	ungrouped	3	.793	.464	.399	.687
637	ungrouped	3	.783	.489	.585	.843
638	ungrouped	3	.643	.882	1.388	1.009
639	ungrouped	3	.594	1.041	1.851	.551
640	ungrouped	3	.558	1.167	.023	.861
641	ungrouped	3	.494	1.410	2.015	.589
642	ungrouped	3	.454	1.577	2.110	.506
643	ungrouped	3	.448	1.606	1.852	1.037
644	ungrouped	3	.381	1.929	.623	1.577
645	ungrouped	3	.352	2.088	2.062	1.057
646	ungrouped	3	.346	2.123	2.159	.926
647	ungrouped	3	.337	2.173	1.287	1.633
648	ungrouped	3	.185	3.377	2.150	1.550
649	ungrouped	3	.155	3.724	2.800	.477
650	ungrouped	3	.069	5.349	2.942	1.278
651	ungrouped	3	.064	5.502	.663	2.549
652	ungrouped	3	.033	6.816	3.340	1.111
653	ungrouped	3	.001	14.618	3.238	3.230
654	ungrouped	2	.974	.053	-.034	-.129
655	ungrouped	2	.905	.199	.328	-.059
656	ungrouped	2	.899	.212	.016	.104
657	ungrouped	2	.863	.294	-.312	-.803
658	ungrouped	2	.806	.432	-.602	-.630
659	ungrouped	2	.666	.811	-.192	.524
660	ungrouped	2	.527	1.282	.860	-1.088
661	ungrouped	1	.401	1.828	-1.763	-.930
662	ungrouped	1	.161	3.651	-2.865	-.370
663	ungrouped	2	.939	.125	.315	-.205
664	ungrouped	2	.385	1.907	.415	-1.672
665	ungrouped	3	.984	.033	.934	.039
666	ungrouped	3	.862	.298	.780	-.321
667	ungrouped	3	.019	7.968	3.343	1.608
668	ungrouped	3	.019	7.968	3.343	1.608
669	ungrouped	3	.019	7.968	3.343	1.608
670	ungrouped	3	.019	7.968	3.343	1.608
671	ungrouped	2	.989	.022	-.042	-.213
672	ungrouped	2	.963	.075	.270	-.362
673	ungrouped	2	.899	.212	-.072	.099
674	ungrouped	2	.840	.350	-.329	.138
675	ungrouped	2	.700	.713	-.338	.419
676	ungrouped	2	.506	1.362	.371	-1.462
677	ungrouped	1	.997	.005	-1.003	.274
678	ungrouped	1	.683	.764	-.714	1.025
679	ungrouped	1	.451	1.594	-2.008	1.035
680	ungrouped	1	.431	1.681	-1.049	1.513
681	ungrouped	3	.746	.586	.248	.633

682	ungrouped	2	.885	.245	-.363	-.016
683	ungrouped	2	.727	.637	-.149	.429
684	ungrouped	2	.711	.682	-.107	.463
685	ungrouped	1	.866	.288	-.601	.516
686	ungrouped	1	.835	.362	-.820	.774
687	ungrouped	2	.997	.007	-.063	-.298
688	ungrouped	2	.991	.017	.038	-.480
689	ungrouped	2	.976	.048	.094	-.160
690	ungrouped	2	.974	.053	-.220	-.435
691	ungrouped	2	.815	.408	-.635	-.459
692	ungrouped	2	.782	.491	-.524	.112
693	ungrouped	2	.771	.519	-.206	.335
694	ungrouped	2	.763	.542	.284	.321
695	ungrouped	2	.747	.584	.214	.376
696	ungrouped	2	.743	.593	-.774	-.349
697	ungrouped	2	.736	.612	-.168	.409
698	ungrouped	2	.720	.656	-.369	-1.080
699	ungrouped	2	.711	.683	-.663	-.855
700	ungrouped	2	.680	.772	-.680	-.917
701	ungrouped	2	.658	.838	-.468	.433
702	ungrouped	2	.592	1.048	-.922	-.810
703	ungrouped	1	.920	.166	-1.327	-.079
704	ungrouped	1	.919	.170	-.727	.478
705	ungrouped	1	.919	.170	-.727	.478
706	ungrouped	1	.919	.170	-.727	.478
707	ungrouped	1	.919	.170	-.727	.478
708	ungrouped	1	.897	.218	-.710	.540
709	ungrouped	1	.801	.443	-1.124	-.444
710	ungrouped	1	.693	.732	-.510	.883
711	ungrouped	1	.693	.732	-.510	.883
712	ungrouped	1	.660	.830	-.713	1.064
713	ungrouped	1	.556	1.172	-.401	1.086
714	ungrouped	3	.978	.045	.764	.043
715	ungrouped	3	.958	.086	.631	.354
716	ungrouped	3	.940	.125	.565	.356
717	ungrouped	3	.912	.185	1.011	.626
718	ungrouped	3	.852	.320	.334	.325
719	ungrouped	3	.775	.509	.628	.878
720	ungrouped	3	.762	.543	1.369	-.344
721	ungrouped	3	.651	.860	1.808	.099
722	ungrouped	3	.469	1.516	.659	1.423
723	ungrouped	3	.439	1.646	2.152	-.007
724	ungrouped	3	.279	2.552	1.959	1.400
725	ungrouped	3	.191	3.310	1.661	1.861
726	ungrouped	3	.065	5.476	2.678	1.722
727	ungrouped	3	.065	5.476	2.678	1.722
728	ungrouped	3	.065	5.476	2.678	1.722
729	ungrouped	3	.065	5.476	2.678	1.722
730	ungrouped	3	.065	5.476	2.678	1.722

731	ungrouped	3	.065	5.476	2.678	1.722
732	ungrouped	3	.065	5.476	2.678	1.722
733	ungrouped	3	.065	5.476	2.678	1.722
734	ungrouped	3	.065	5.476	2.678	1.722
735	ungrouped	3	.065	5.476	2.678	1.722
736	ungrouped	3	.065	5.476	2.678	1.722
737	ungrouped	3	.065	5.476	2.678	1.722
738	ungrouped	3	.065	5.476	2.678	1.722
739	ungrouped	3	.065	5.476	2.678	1.722
740	ungrouped	3	.037	6.589	-.100	2.583
741	ungrouped	3	.003	11.809	3.660	2.246
742	ungrouped	2	.997	.005	-.074	-.343
743	ungrouped	2	.983	.034	.112	-.212
744	ungrouped	2	.963	.076	.015	-.082
745	ungrouped	2	.949	.104	.319	-.342
746	ungrouped	2	.946	.111	-.211	-.096
747	ungrouped	2	.926	.155	-.038	.035
748	ungrouped	2	.922	.162	-.017	.046
749	ungrouped	2	.842	.345	.459	.004
750	ungrouped	2	.811	.418	-.585	-.639
751	ungrouped	2	.801	.443	.366	.197
752	ungrouped	2	.728	.635	.659	-.799
753	ungrouped	2	.636	.904	.236	-1.277
754	ungrouped	2	.515	1.326	-.370	-1.448
755	ungrouped	2	.361	2.036	-.415	-1.723
756	ungrouped	2	.276	2.573	-1.039	-1.582
757	ungrouped	3	.974	.053	.672	.136
758	ungrouped	3	.958	.085	.622	.332
759	ungrouped	3	.849	.328	1.200	.694
760	ungrouped	3	.818	.403	.468	.689
761	ungrouped	3	.604	1.008	.150	.894
762	ungrouped	3	.043	6.305	1.038	2.720
763	ungrouped	2	.955	.093	-.179	-.107
764	ungrouped	2	.818	.402	-.073	.274
765	ungrouped	1	.861	.298	-.554	.454
766	ungrouped	1	.819	.400	-1.571	-.137
767	ungrouped	3	.999	.002	.930	.191
768	ungrouped	3	.910	.189	.823	.644
769	ungrouped	3	.892	.229	1.240	-.111
770	ungrouped	3	.837	.357	1.158	.747
771	ungrouped	3	.723	.648	1.618	.555
772	ungrouped	3	.720	.657	.588	.966
773	ungrouped	3	.710	.684	1.139	1.002
774	ungrouped	3	.710	.685	1.478	.795
775	ungrouped	3	.654	.850	1.057	-.693
776	ungrouped	3	.541	1.230	1.987	.364
777	ungrouped	3	.518	1.315	1.929	.695
778	ungrouped	3	.497	1.399	1.127	1.372
779	ungrouped	3	.456	1.572	.886	1.468

780	ungrouped	3	.385	1.908	1.338	1.520
781	ungrouped	3	.384	1.916	2.106	.873
782	ungrouped	3	.322	2.269	2.192	.969
783	ungrouped	3	.261	2.683	-.085	1.531
784	ungrouped	3	.224	2.988	.038	1.719
785	ungrouped	3	.202	3.197	2.290	1.324
786	ungrouped	3	.173	3.512	2.538	1.103
787	ungrouped	3	.061	5.604	.946	2.580
788	ungrouped	3	.014	8.525	1.493	3.070
789	ungrouped	3	.009	9.399	3.049	2.389
790	ungrouped	2	.941	.123	-.108	-.022
791	ungrouped	2	.844	.339	.578	-.345
792	ungrouped	2	.826	.381	-.591	-.166
793	ungrouped	2	.808	.427	.332	.204
794	ungrouped	2	.767	.531	-.518	.160
795	ungrouped	2	.523	1.298	-1.085	-.715
796	ungrouped	2	.360	2.044	-.165	1.064
797	ungrouped	2	.341	2.154	-1.184	-1.229
798	ungrouped	2	.245	2.813	-.229	-2.019
799	ungrouped	1	.976	.049	-1.136	.014
800	ungrouped	1	.944	.114	-.779	.009
801	ungrouped	1	.788	.476	-1.340	-.408
802	ungrouped	1	.585	1.072	-1.534	1.129
803	ungrouped	1	.415	1.757	-2.257	.757
804	ungrouped	1	.409	1.787	-.983	1.552
805	ungrouped	1	.383	1.917	-2.177	-.582
806	ungrouped	1	.176	3.479	-.365	1.953
807	ungrouped	1	.032	6.910	-3.665	-.009
808	ungrouped	3	.999	.002	.903	.167
809	ungrouped	3	.966	.068	.927	-.045
810	ungrouped	3	.938	.128	1.130	-.051
811	ungrouped	3	.303	2.389	2.086	1.190
812	ungrouped	3	.019	7.968	3.343	1.608
813	ungrouped	3	.019	7.968	3.343	1.608
814	ungrouped	3	.019	7.968	3.343	1.608
815	ungrouped	3	.019	7.968	3.343	1.608
816	ungrouped	3	.019	7.968	3.343	1.608
817	ungrouped	3	.003	11.962	3.904	1.908
818	ungrouped	2	.871	.277	-.219	.124
819	ungrouped	2	.835	.361	.511	-.667
820	ungrouped	2	.779	.500	.304	.280
821	ungrouped	2	.778	.503	.320	-.987
822	ungrouped	2	.575	1.108	.702	-1.137
823	ungrouped	2	.480	1.470	.435	-1.487
824	ungrouped	3	.992	.015	.891	.090
825	ungrouped	3	.909	.190	.455	.171
826	ungrouped	3	.772	.516	.271	.581
827	ungrouped	3	.723	.648	.163	.563
828	ungrouped	3	.723	.648	.163	.563

829	ungrouped	3	.689	.745	.222	.761
830	ungrouped	3	.444	1.624	1.543	1.307
831	ungrouped	3	.249	2.784	1.063	1.873
832	ungrouped	2	.968	.064	.174	-.538
833	ungrouped	2	.960	.081	.051	-.078
834	ungrouped	2	.945	.112	.314	-.463
835	ungrouped	2	.909	.190	-.315	-.051
836	ungrouped	2	.909	.191	.257	-.006
837	ungrouped	2	.901	.208	-.071	.094
838	ungrouped	2	.868	.283	-.526	-.256
839	ungrouped	2	.842	.345	-.103	.222
840	ungrouped	2	.809	.424	-.194	.266
841	ungrouped	2	.637	.901	-.664	-1.039
842	ungrouped	2	.585	1.071	-.820	-.992
843	ungrouped	2	.510	1.348	-1.094	-.757
844	ungrouped	2	.250	2.773	-1.382	-1.292
845	ungrouped	1	.898	.216	-1.243	.637
846	ungrouped	1	.775	.509	-1.709	-.046
847	ungrouped	1	.306	2.370	-.184	1.492
848	ungrouped	3	.873	.272	.525	.588
849	ungrouped	3	.750	.576	.560	.898
850	ungrouped	3	.065	5.476	2.678	1.722
851	ungrouped	3	.065	5.476	2.678	1.722
852	ungrouped	3	.065	5.476	2.678	1.722
853	ungrouped	3	.065	5.476	2.678	1.722
854	ungrouped	2	.993	.014	.065	-.453
855	ungrouped	2	.963	.076	.142	-.122
856	ungrouped	2	.949	.105	-.216	-.602
857	ungrouped	2	.713	.676	-.550	.258
858	ungrouped	2	.660	.830	.508	-1.110
859	ungrouped	2	.505	1.365	-.089	.809
860	ungrouped	2	.356	2.066	.042	-1.793

Appendix D

Source Molecular Corporation
Human and Bovine *Enterococcus* and
Human and Bovine and Equine *Bacteroidetes* Methods

Human Enterococcus DNA Analytical Method

For each sample, 100 ml of water was filtered through a 0.45 micron membrane filter and placed on mEI agar. The samples were incubated for 24 hours. Each filter was removed, placed in a buffer and vortexed vigorously. Once the buffer was spun to pellet the bacteria, the supernatant was removed and the pellet was resuspended in a small volume of water. DNA extraction was prepared using the Qiagen DNA extraction kit, as per manufacturer's instructions. Five micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl2, 150 mM dNTP, and 0.3 mM of each primer. An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 95oC for 15 minutes (to lyse cells and activate polymerase), followed by 35 cycles of 94oC for 1 minute, 55oC for 1 minute, and 72oC for 1 minute and a final extension at 72oC for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Cambrex, Inc.) and visualized under UV light.

Enterococci are a subgroup of Fecal Streptococci and are characterized by their ability to grow in 6.5% sodium chloride, at low and elevated temperatures (10oC and 45oC), and at elevated pH (9.5). These microorganisms have been used as indicators of fecal pollution for many years and have been especially valuable in the marine environment and recreational waters as indicators of potential health risks and swimming-related gastroenteritis.[1] *Enterococci* are benign bacteria when they reside in their normal habitat such as the gastrointestinal tracts of human or animals. Outside of their normal habitat, *Enterococci* are pathogenic causing urinary tract and wound infections, and life-threatening diseases such as bacteraemia, endocarditis, and meningitis. *Enterococci* easily colonize open wounds and skin ulcers. Compounding their pathogenesis, *Enterococci* are also some of the most antibiotic resistant bacteria, particularly from human sources. Studies have shown that certain strains of *Enterococci* are resistant to expensive and potent antibiotics such as vancomycin. This is particularly worrisome for the medical community since these antibiotics are given as a last resort to fight severe bacterial infections. Several intrinsic features of the *Enterococcus* genus allow it to survive for extended periods of time, leading to its extended survivability and diffusion. For example, *Enterococci* have been shown to survive for 30 minutes at 60°C and persist in the presence of detergents. As such, the inherent ruggedness of *Enterococcus* confers it a strong tolerance to many classes of antibiotics. The Human *Enterococcus* ID™ service is designed around the principle that certain strains of the *Enterococcus* genus are specific to humans.[2,3,4] These *Enterococci* can be used as indicators of human fecal contamination. Strains of *Enterococcus faecium*, *Enterococcus faecalis* and yellow-pigmented *Enterococci* have been shown to be from human sources.[2,3,4] Within these Enterococcus spp. are genes associated with *Enterococci* that are specific to humans.[5] The Human *Enterococcus* ID™ service targets the esp human gene biomarker in *Enterococcus faecium*.[6] One of the advantages of the Human *Enterococcus* ID™ service is that the entire population of *Enterococci* of the selected portion of the water sample is screened. As such, this method avoids the randomness effect of selecting isolates off a petri dish. This is a particular advantage for highly contaminated water systems with

potential multiple sources of fecal contamination. Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected. Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis. The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the human gene biomarker from *Enterococcus faecium*. These banding patterns confirm or negate the presence of the *Enterococci* human gene biomarker. As such, the banding patterns provide a reliable indicator of human fecal contamination. To strengthen the validity of the results, the Human *Enterococcus* ID™ service should be combined with other DNA analytical services such as the Human *Bacteroidetes* ID™ and Human Fecal Virus ID™ services.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.
[2] Scott, T.M., T.M. Jenkins, J. Lukasik, and J.B. Rose. 2005. **Potential Use of a Host Associated Molecular Marker in *Enterococcus faecium* as an Index of Human Fecal Pollution.** Environ. Sci. Technol. 39: 283-287.
[3] Bahirathan ML, Puente L, Seyfried P. 1998. **Use of yellow-pigmented enterococci as a specific indicator of human and nonhuman sources of faecal pollution.** Can J Microbiol 44:1066-1071.
[4] Quednau, M., Ahrne, S., Molin, G. **Genomic Relationships between Enterococcus faecium Strains from Different Sources and with Different Antibiotic Resistance Profiles Evaluated by Restriction Endonuclease Analysis of Total Chromosomal DNA Using EcoRI and PvuII.** Appl. Environ. Microbiol. 1999 65: 1777-1780.
[5] Hammerum, A.M., and L.B. Jensen. 2002. **Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark.** J. Clin. Microbiol. 40: 4396.
[6] Soule, Marilyn, Kuhn, Edward, Loge, Frank, Gay, John, Call, Douglas R. **Using DNA Microarrays To Identify Library-Independent Markers for Bacterial Source Tracking** Appl. Environ. Microbiol. 2006 72: 1843-1851.
[7] **EPA Method 1600 (modified): Membrane Filter Test Method for Enterococci In Water (1997).**

Bovine Enterococcus DNA Analytical Method

For each sample, 150 ml of water was filtered through a 0.45 micron membrane filter and placed on mEI agar. The samples were incubated for 24 hours. Each filter was removed, placed in a buffer and vortexed vigorously. Once the buffer was spun to pellet the bacteria, the supernatant was removed and the pellet was resuspended in a small volume of water. DNA extraction was prepared using the Qiagen DNA extraction kit, as per manufacturer's instructions. Five micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl2, 150 mM dNTP, and 0.3 mM of each primer. An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 95^{o}C for 15 minutes (to lyse cells and activate polymerase), followed by 35 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute, and 72^{o}C for 1 minute and a final extension at 72^{o}C for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Cambrex, Inc.) and visualized under UV light.

Enterococci are a subgroup of Fecal *Streptococci* and are characterized by their ability to grow in 6.5% sodium chloride, at low and elevated temperatures (10^{o}C and 45^{o}C), and at elevated pH (9.5). These microorganisms have been used as indicators of fecal pollution for many years and have been especially valuable in the marine environment and recreational waters as indicators of potential health risks andswimming-related gastroenteritis.[1,2,3] *Enterococci* are benign bacteria when they reside in their normal habitat such as the gastrointestinal tracts of human or animals. Outside of their normal habitat, *Enterococci* are pathogenic causing urinary tract and wound infections, and life-threatening diseases such as bacteraemia, endocarditis, and meningitis. *Enterococci* easily colonize open wounds and skin ulcers. Compounding their pathogenesis, *Enterococci* are also some of the most antibiotic resistant bacteria.[4,5] Studies have shown that certain strains of *Enterococci* are resistant to expensive and potent antibiotics such as vancomycin. This is particularly worrisome for the medical community since these antibiotics are given as a last resort to fight severe bacterial infections. Several intrinsic features of the *Enterococcus* genus allow it to survive for extended periods of time, leading to its extended survivability and diffusion. For example, *Enterococci* have been shown to survive for 30 minutes at 60°C and persist in the presence of detergents. As such, the inherent ruggedness of *Enterococcus* confers it a strong tolerance to many classes of antibiotics. The Cow Enterococcus IDTM service is designed around the principle that certain DNA sequences contained within strains of the *Enterococcus* genus are specific to cattle. These *Enterococci* sequences can be used as indicators of cattle fecal contamination. 6 Strains of *Enterococcus hirae* and *Enterococcus mundtii* have been shown to be from cattle and other ruminant sources.6 The Cow Enterococcus IDTM service targets the cattle gene biomarker in *Enterococcus hirae*. One of the advantages of the Cow Enterococcus IDTM service is that the entire population of *Enterococci* of the selected portion of the water sample is screened. As such, this method avoids the randomness effect of selecting isolates off a petri dish. Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished

with small pieces of DNA called primers that are complementary and specific to the genomes to be detected. Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis. The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the cattle gene biomarker from *Enterococcus hirae.* These banding patterns confirm or negate the presence of the *Enterococci* cattle gene biomarker. As such, the banding patterns provide a reliable indicator of cattle fecal contamination. To strengthen the validity of the results, the Cow *Enterococcus* ID™ service should be combined with other DNA analytical services such as the Cow *Bacteroidetes* ID™ and Cow Fecal Virus ID™ services.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.
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[4] Quednau, M., Ahrne, S., Molin, G. **Genomic Relationships between Enterococcus faecium Strains from Different Sources and with Different Antibiotic Resistance Profiles Evaluated by Restriction Endonuclease Analysis of Total Chromosomal DNA Using EcoRI and PvuII.** Appl. Environ. Microbiol. 1999 65: 1777-1780.
[5] Hammerum, A.M., and L.B. Jensen. 2002. **Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark.** J. Clin. Microbiol. 40: 4396.
[6] Soule, Marilyn, Kuhn, Edward, Loge, Frank, Gay, John, Call, Douglas R. **Using DNA Microarrays To Identify Library-Independent Markers for Bacterial Source Tracking** Appl. Environ. Microbiol. 2006 72: 1843-1851.
[7] **EPA Method 1600 (modified): Membrane Filter Test Method for Enterococci In Water (1997).**

Human Bacteroidetes DNA Analytical Method

The water samples were filtered through 0.45 micron membrane filters. The filters were placed in separate 15-ml disposable centrifuge tubes containing 2 ml of lysis buffer. DNA extraction was prepared using a Qiagen DNA extraction kit, as per manufacturer's instructions. Two micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl2, 150 mM dNTP, and 0.3 mM of each primer.
An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 25 cycles of 94°C for 30 s, appropriate annealing temperature for 30 s, and 72°C for 1 min followed by a final 6-min extension at 72°C. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Biowhittaker, Inc.) and visualized under UV light.

The phylum *Bacteroidetes* is composed of three large groups of bacteria with the best-known category being *Bacteroidaceae*. This family of gram-negative bacteria is found primarily in the intestinal tracts and mucous membranes of warm-blooded animals and is sometimes considered pathogenic. Comprising *Bacteroidaceae* are the genus *Bacteroides* and *Prevotella*. The latter genus was originally classified within the former (i.e. *Bacteroides*), but since the 1990's it has been classified in a separate genus because of new chemical and biochemical findings. *Bacteroides* and *Prevotella* are gram-negative, anaerobic, rod-shaped bacteria that inhabitant of the oral, respiratory, intestinal, and urogenital cavities of humans, animals, and insects. They are sometimes pathogenic. Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[1] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems. This is a particularly strong reference point when trying to determine recent outbreaks in fecal pollution. They are also more abundant in feces of warm-blooded animals than *E. coli* and *Enterococci*. Furthermore, these latter two organisms are facultative anaerobes and as such they can be problematic for monitoring purposes since it has been shown that they are able to proliferate in soil, sand and sediments. The Human Bacteroidetes IDTM service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[2,3,4,5,6] Furthermore, certain categories of *Bacteroidetes* have been shown to be predominately found in humans. Within these *Bacteroidetes*, certain strains of the *Bacteroides* and *Prevotella* genus have been found to be specific to humans.[2,3] As such, these bacterial strains can be used as indicators of human fecal contamination. One of the advantages of the Human Bacteroidetes IDTM service is that the entire water is sampled and filtered for fecal *Bacteroidetes*. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates off a petri dish. This is a particular advantage for highly contaminated water systems with potential multiple sources of fecal contamination. Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected.

Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis. The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the human gene biomarker from the *Bacteroides* and *Prevotella* genus. These banding patterns confirm or negate the presence of the fecal *Bacteroidetes* human gene biomarker. As such, the banding patterns provide a reliable indicator of human fecal contamination. To strengthen the validity of the results, the Human Bacteroidetes IDTM service should be combined with other DNA analytical services such as the Human Enterococcus IDTM and Human Fecal Virus ID™ services.

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[2] Bernhard, A.E., and K.G. Field (2000a). **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Applied and Environmental Microbiology, 66: 1,587-1,594.
[3] Bernhard, A.E., and K.G. Field (2000b). **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Applied and Environmental Microbiology, 66: 4,571-4,574.
[4] Kreader, C.A. (1995). **Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution.** Applied and Environmental Microbiology, 61: 1,171-1,179.
[5] Kreader, C.A. (1998). **Persistence of PCR-detectable Bacteroides distasonis from human feces in river water.** Applied and Environmental Microbiology, 64: 4,103-4,105.
[6] Dick, Linda K., Field, Katharine G.**Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes.** Appl. Environ. Microbiol. 2004 70: 5695-5697.

Bovine Bacteroidetes DNA Analytical Method

The water samples were filtered through 0.45 micron membrane filters. The filters were placed in separate 15-ml disposable centrifuge tubes containing 2 ml of lysis buffer. DNA extraction was prepared using a Qiagen DNA extraction kit, as per manufacturer's instructions. Two micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl2, 150 mM dNTP, and 0.3 mM of each primer.
An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 25 cycles of 94°C for 30 s, appropriate annealing temperature for 30 s, and 72°C for 1 min followed by a final 6-min extension at 72°C. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Biowhittaker, Inc.) and visualized under UV light.

The phylum *Bacteroidetes* is composed of three large groups of bacteria with the best-known category being *Bacteroidaceae*. This family of gram-negative bacteria is found primarily in the intestinal tracts and mucous membranes of warm-blooded animals and is sometimes considered pathogenic. Comprising *Bacteroidaceae* are the genus *Bacteroides* and *Prevotella*. The latter genus was originally classified within the former (i.e. *Bacteroides*), but since the 1990's it has been classified in a separate genus because of new chemical and biochemical findings. *Bacteroides* and *Prevotella* are gram-negative, anaerobic, rod-shaped bacteria that inhabitant of the oral, respiratory, intestinal, and urogenital cavities of humans, animals, and insects. They are sometimes pathogenic. Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[1] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems. This is a particularly strong reference point when trying to determine recent outbreaks in fecal pollution. They are also more abundant in feces of warm-blooded animals than *E. coli* and *Enterococci*. Furthermore, these latter two organisms are facultative anaerobes and as such they can be problematic for monitoring purposes since it has been shown that they are able to proliferate in soil, sand and sediments. The Cow Bacteroidetes IDTM service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[2,3,4,5,6] Furthermore, certain categories of *Bacteroidetes* have been shown to be predominately detected in cattle. Within these *Bacteroidetes*, certain strains of the *Bacteroides* and *Prevotella* genus have been found in cattle.[2,3,5] As such, these bacterial strains can be used as indicators of cattle fecal contamination. One of the advantages of the Cow Bacteroidetes IDTM service is that the entire water is sampled and filtered for fecal *Bacteroidetes*. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates off a petri dish. This is a particular advantage for highly contaminated water systems with potential multiple sources of fecal contamination. Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected. Through a heating process called thermal cycling, the double stranded DNA is denatured

and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis. The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the cattle gene biomarker from the *Bacteroides* and *Prevotella* genus. These banding patterns confirm or negate the presence of the fecal *Bacteroidetes* cattle gene biomarker. As such, the banding patterns can be a good indicator of cattle fecal contamination. Nonetheless, in order to strengthen the validity of the results, the Cow Bacteroidetes ID™ service should be combined with other DNA analytical tests such as the Cow Enterococcus ID™ and Cow Fecal Virus ID™ services to further confirm the results.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.
[2] Bernhard, A.E., and K.G. Field (2000a). **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Applied and Environmental Microbiology, 66: 1,587-1,594.
[3] Bernhard, A.E., and K.G. Field (2000b). **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Applied and Environmental Microbiology, 66: 4,571-4,574.
[4] Kreader, C.A. (1995). **Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution.** Applied and Environmental Microbiology, 61: 1,171-1,179.
[5] Kreader, C.A. (1998). **Persistence of PCR-detectable Bacteroides distasonis from human feces in river water.** Applied and Environmental Microbiology, 64: 4,103-4,105.
[6] Dick, Linda K., Field, Katharine G.**Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes.** Appl. Environ. Microbiol. 2004 70: 5695-5697.

Equine Bacteroidetes DNA Analytical Method

The water samples were filtered through 0.45 micron membrane filters. The filters were placed in separate 15-ml disposable centrifuge tubes containing 2 ml of lysis buffer. DNA extraction was prepared using a Qiagen DNA extraction kit, as per manufacturer's instructions. Two micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl2, 150 mM dNTP, and 0.3 mM of each primer.
An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 25 cycles of 94°C for 30 s, appropriate annealing temperature for 30 s, and 72°C for 1 min followed by a final 6-min extension at 72°C. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Biowhittaker, Inc.) and visualized under UV light.

The phylum *Bacteroidetes* is composed of three large groups of bacteria with the best-known category being *Bacteroidaceae*. This family of gram-negative bacteria is found primarily in the intestinal tracts and mucous membranes of warm-blooded animals and is sometimes considered pathogenic. Comprising *Bacteroidaceae* are the genus *Bacteroides* and *Prevotella*. The latter genus was originally classified within the former (i.e. *Bacteroides*), but since the 1990's it has been classified in a separate genus because of new chemical and biochemical findings. *Bacteroides* and *Prevotella* are gram-negative, anaerobic, rod-shaped bacteria that inhabitant of the oral, respiratory, intestinal, and urogenital cavities of humans, animals, and insects. They are sometimes pathogenic. Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[1] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems. This is a particularly strong reference point when trying to determine recent outbreaks in fecal pollution. They are also more abundant in feces of warm-blooded animals than *E. coli* and *Enterococci*. Furthermore, these latter two organisms are facultative anaerobes and as such they can be problematic for monitoring purposes since it has been shown that they are able to proliferate in soil, sand and sediments. The Horse Bacteroidetes ID™ service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[2,3,4,5,6] Furthermore, certain categories of *Bacteroidetes* have been shown to be predominately detected in horse. Within these *Bacteroidetes*, certain strains of the *Bacteroides* and *Prevotella* genus have been found in horse.[2,3,5,6] As such, these bacterial strains can be used as indicators of horse fecal contamination. One of the advantages of the Horse Bacteroidetes ID™ service is that the entire water is sampled and filtered for fecal *Bacteroidetes*. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates off a petri dish. This is a particular advantage for highly contaminated water systems with potential multiple sources of fecal contamination. Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected. Through a heating process called thermal cycling, the double stranded DNA is denatured

and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis. The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the horse gene biomarker from the *Bacteroides* and *Prevotella* genus. These banding patterns confirm or negate the presence of the fecal *Bacteroidetes* horse gene biomarker. As such, the banding patterns can be a good indicator of horse fecal contamination. Nonetheless, in order to strengthen the validity of the results, the Horse Bacteroidetes ID™ service should be combined with other DNA analytical services such as the E. coli ID™ service.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.
[2] Bernhard, A.E., and K.G. Field (2000a). **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Applied and Environmental Microbiology, 66: 1,587-1,594.
[3] Bernhard, A.E., and K.G. Field (2000b). **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Applied and Environmental Microbiology, 66: 4,571-4,574.
[4] Kreader, C.A. (1995). **Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution.** Applied and Environmental Microbiology, 61: 1,171-1,179.
[5] Kreader, C.A. (1998). **Persistence of PCR-detectable Bacteroides distasonis from human feces in river water.** Applied and Environmental Microbiology, 64: 4,103-4,105.
[6] Dick, Linda K., Field, Katharine G.**Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes.** Appl. Environ. Microbiol. 2004 70: 5695-5697.

[1] Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.
[2] Bernhard, A.E., and K.G. Field (2000a). **Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes.** Applied and Environmental Microbiology, 66: 1,587-1,594.
[3] Bernhard, A.E., and K.G. Field (2000b). **A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA.** Applied and Environmental Microbiology, 66: 4,571-4,574.
[4] Kreader, C.A. (1995). **Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution.** Applied and Environmental Microbiology, 61: 1,171-1,179.
[5] Kreader, C.A. (1998). **Persistence of PCR-detectable Bacteroides distasonis from human feces in river water.** Applied and Environmental Microbiology, 64: 4,103-4,105.
[6] Dick, Linda K., Field, Katharine G.**Rapid Estimation of Numbers of Fecal Bacteroidetes by Use of a Quantitative PCR Assay for 16S rRNA Genes.** Appl. Environ. Microbiol. 2004 70: 5695-5697.

Appendix F

Quality Assurance Plan:
Upper Fish River Bacterial Source Tracking Project

Quality Assurance Plan:
Upper Fish River Bacterial Source Tracking Project

1.0 *E. coli* Sampling

Data collected as part of the project follows the standard Alabama Water Watch (AWW) standard operating procedures and the sampling quality assurance plan as adopted in 1999. The Project Coordinator and volunteer E. coli monitor are certified bacteria monitor as specified by the AWW standard operating procedures and the sampling quality assurance plan. The AWW Bacteria Sampling Quality Assurance Plan is approved by the US Environmental Protection Agency and copies of the plan are available from the AWW office at Auburn University or at the AWW website.

2.0 *E. coli* Media

2.1 Coliscan Easygel®. E. coli monitoring supplies are purchased directly from Micrology Laboratories, LLC (Goshen, IL) and are stored and used according to manufacturing guidelines. Examples of Micrology Labs quality checks of Coliscan Easygel® media are included in Appendix A. Performance of the media is indirectly checked using sterile sample water. With each media lot, media is incubated without addition of sample water. Also, an aliquot of sample water is sterilized and plated alongside original sample water. Growth conditions are recorded. No E. coli test strains are used on media batches but batch quality information is available at request from the manuafacturer (Appendix A).

2.2 Mueller-Hinton Media. Preparation requirements are established by the manufacturer, Becton-Dickinson (Franklin Lakes, NJ), and followed. Manufacturer's specifications are included in Appendix D. Performance is monitored using commercial strains purchased from the media manufacturer and recorded.

3.0 Temperature

3.1 Thermometers. In each incubator, temperature is determined by a LaMotte (Chestertown, MD) precision, **NON-MERCURY** thermometer with engraved graduations over the full range of -5° to 45°C in 0.5° increments. Accuracy of LaMotte thermometer is determined annually using a traceable digital thermometer.

3.2 Traceable Thermometer. Accuracy of regular thermometers is determined using a Fisher Brand traceable digital thermometer. Digital thermometer is returned to manufacturer for annual calibration check. Calibration checks are maintained on file.

3.3 Incubation Temperature: Coliscan Easygel®. Incubation requirements listed in the AWW Bacteria Sampling Quality Assurance Plan are met by all incubations carried out as part of this project. The Coliscan Easygel® growth media used in this project does not require tightly controlled temperature tolerances (30-37°C according to manufacturers guidelines) to effectively express the color indicator produced by E. coli growth.

3.4 Incubation Temperature: Mueller-Hinton Media. Incubation requirements are established by the manufacturer, Becton-Dickinson (Franklin Lakes, NJ), and followed. Manufacturer's specifications are included in Appendix D. Incubation temperature is monitored by National Institutes of Standards-traceable thermometer.

4.0 Sterilization

4.1. Coliscan Easygel®. Sterility of media lots is monitored by incubating uninoculated media under the same conditions as inoculated media. Also, media is inoculated with sterilized sample water and no growth monitored.

4.2 Mueller-Hinton. Sterility of media and utensils is achieved with a steam sterilizer operating at 121 degrees celsius and 15 psi. Sterility is monitored by checking sterilized media for contamination and by using sterile technique in handling bacteria.

5.0 Rainfall Monitoring

5.1 Electronic Rain Gauges. The electronic rain gauges are Oregon Scientific™ Model RGR682. Both the outside bucket and inside receiving units is powered by batteries. Installation, operation and maintenance are performed according to manufacturer's specifications. Specification for gauges and instruction sheets provided to each volunteer rainfall monitor are included in Appendix B and C, respectively.

Appendix A.
Certificate of Quality Control for Coliscan Easygel® provided by Micrology Laboratories, LLC.

Quality Control Certificate
Certificate of Analysis

Product **Coliscan Easygel**

Product Number **25001**

Lot number **3A149**

Representative samples have been tested by the quality control laboratory. Procedures and results are listed below.

Procedures:

Inoculum from the listed cultures was introduced on/in selected medium samples to test performance. The medium was also examined to verify appropriate physical characteristics.

Results:

Test cultures	Growth	Reaction
Escherichia coli	Excellent	Blue/purple
Enterobacter aerogenes	Excellent	Red colony
Salmonella typhimurium	Very good	Colorless
Staphylococcus aureaus	Inhibited	Inhibited

Physical Characteristics:

Appearance: Tan liquid
Strerility: Inspected after 1, 2 and 5 day incubation
pH: 7.4 ± 0.2 at 25 C

Manufactured by: Micrology Laboratories, LLC.
Goshen, Indiana USA
Phone 574-533-3351

Appendix B.
Oregon Scientific™ Model RGR682 Electronic Rain Gauge Specification Sheet

Oregon SCIENTIFIC

Wireless Rain Gauge with 10-Day Rainfall Memory and Digital Clock
MODEL: RGR682

USER MANUAL

INTRODUCTION

Thank you for selecting the Oregon Scientific™ Wireless Rain Gauge RGR682.

NOTE Keep this manual handy as you use your new product. It contains practical step-by-step instructions, as well as technical specifications and warnings you should know about.

MAIN UNIT OVERVIEW

FRONT VIEW / LCD DISPLAY

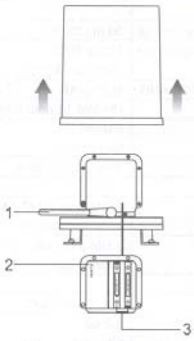
1. Total rainfall indicator
2. Rain gauge RF reception status
3. Number of days of rainfall history
4. Rain alarm indicator
5. Clock alarm indicator
6. Clock AM / PM indicator
7. Rain collector battery low indicator
8. Total rainfall measurements indicator
9. Rainfall history
10. SINCE indicator
11. Main unit battery low indicator
12. Calendar clock / clock alarm / start date of total rainfall record

BACK VIEW

1. **RAIN ON / OFF button:** Enables or disables the rainfall alarm
2. **RAIN button:** Press to display rain alarm (default 30mm). Press again to return to the rainfall display
3. **MODE button:** Toggles display between clock with seconds, clock with weekday, calendar and alarm time
4. **ALARM button:** Turns daily alarm off
5. **SINCE button:** Toggles between starting date of total rainfall calculation and clock; Press and hold to reset the total rainfall counter to start again
6. **HISTORY / UP button:** Displays rainfall history in normal mode; Increases a value in rainfall alarm / calendar clock setting mode
7. **DOWN button:** Decreases a value in rainfall alarm / calendar clock setting mode
8. Battery compartment
9. **SEARCH button:** Search for the rain collector
10. **IN / MM button:** Selects between inch and millimeter measurements
11. **RESET hole:** Returns all settings to their default values

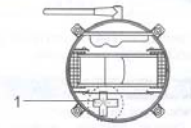
RAIN COLLECTOR OVERVIEW

SIDE VIEW



1. **Antenna:** Transmits radio signal to main unit
2. **RESET** hole
3. Battery compartment

TOP VIEW

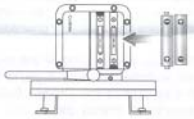


1. **Cross:** Checks the leveling of the rain collector

BATTERY INSTALLATION

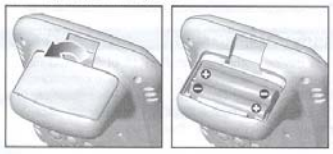
RAIN COLLECTOR

Remove battery compartment and insert batteries according to polarities.



MAIN UNIT

Open the battery compartment and insert batteries matching the polarities (+ / -).



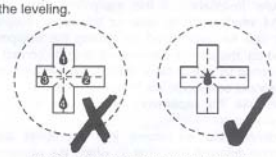
indicates the batteries are low

NOTE Do not use rechargeable batteries. We recommend that you use alkaline batteries with this product for longer usage.

UNIT	LOCATION
Main	Clock area
Remote	Rainfall history area

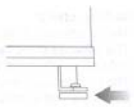
SETUP

1. Mount the rain collector on a level surface, positioning it within effective range (30 m / 100 ft) of the main unit.
2. Put drops of water on the cross at the base to check the leveling.



Water staying at position 1-4 means the gauge is not leveled.

3. Use the metal ring to adjust the leveling of the rain collector.



4. Remove the fiber tape from around the bucket assemblies.

NOTE Each time the battery in the main unit is changed, repeat rain collector setup steps 1-4 before use.

RAINFALL

Today's rainfall appears on the 1st line of the display and total rainfall is shown on the 2nd line. Press **IN / MM** to toggle between inches and millimeters as the unit of measurement.

RAINFALL HISTORY

The rainfall history is displayed on the second line of the LCD display. The main unit can record and store to nine days of rainfall.

To display the record for a particular day:
Use **HISTORY / UP** to toggle between daily rainfall and rainfall history over the past 9 days. The day of the record will be displayed with a minus (-) sign. Zero (0) means the record is for the current day.

To clear the current day rainfall:
Press and hold **SINCE** for two seconds. Note that this will also clear the total rainfall record.

CAUTION Other sensors using the 433 MHz transmission frequency may influence the rainfall reading. Please avoid placing those sensors too close to the unit.

TOTAL RAINFALL

The total rainfall is displayed on the 1st line of the LCD display.

To display the commencing date of the total rainfall record, press **SINCE**. The date will appear on the bottom line of the display.

To clear the existing commencing date and reset it to start again, press and hold **SINCE**. The total rainfall and today rainfall will be reset to zero and the unit will start again to collect the rainfall data.

RAINFALL ALARM

The rain alarm value can be displayed at any time by pressing **RAIN**.

To set the rainfall alarm:
1. Press and hold **RAIN** for two seconds.
2. Use **HISTORY / UP** or **DOWN** to set the desired value.
3. Press **RAIN** to confirm. The alarm will be activated.

The rain alarm indicator will light up.

The alarm will go off for one minute when the rainfall reaches the preset value and the rain alarm indicator will flash. A reminder beep will be emitted every minute. Once the rain alarm is on the indicator will not stop flashing unless **RAIN** is pressed or the rain alarm value is changed. To stop the alarm and reminder beep, press any button or change the rainfall alarm value.

To toggle the alarm **ON / OFF**, press **RAIN ON / OFF**.

DISCONNECTED SIGNALS

If without obvious reason the main unit display goes blank, press and hold **SEARCH** for 2 seconds to enforce an immediate search of the rain collector.

LCD SYMBOL	DESCRIPTION
RAIN	No signal
RAIN	Searching for signal
RAIN → RAIN → RAIN	Signal connected

If that fails, check:

- The remote rain collector is still in place.
- The batteries of the main unit and rain collector have not run out of power. Replace them if necessary.
- The transmission is within range and path is clear of obstacles and interference. Shorten the distance if necessary.

Then press **SEARCH** again.

CALENDAR CLOCK

The calendar clock is displayed on the bottom line of the display. Use **MODE** to toggle between clock, calendar and daily alarm.

To set the clock:

1. Press **MODE** to display the clock or calendar.
2. Press and hold **MODE** for two seconds and use **HISTORY / UP** or **DOWN** to set the desired clock or calendar value.
3. Press **MODE** and repeat from step 2 to complete all settings.
4. Press **MODE** to confirm.

To set the daily alarm:

1. Press **MODE** to display the daily alarm. The daily alarm will be activated automatically. The daily alarm indicator will light up.
2. Press and hold **MODE** for two seconds and use **HISTORY / UP** or **DOWN** to set the desired value. Press and hold for faster increments.
3. Press **MODE** and repeat from step 2 to complete all settings.

When active, the daily alarm will go off for one minute at the set time and the daily alarm indicator will flash.

To stop the alarm:
Press any button. The alarm is still active and will go off at the set time the following day.

To deactivate the daily alarm all together:
Press **ALARM**.

RESET

Press **RESET** to return the unit to the default settings.

PRECAUTIONS

This product is engineered to provide years of satisfactory service if you handle it carefully. Here are a few precautions:

- Do not immerse the unit in water. If you spill liquid over it, dry it immediately with a soft, lint-free cloth.
- Do not clean the unit with abrasive or corrosive materials. They may scratch the plastic parts and corrode the electronic circuit.
- Do not subject the unit to excessive force, shock, dust, temperature or humidity, which may result in malfunction, shorter electronic life span, damaged battery and distorted parts.
- Do not tamper with the unit's internal components. Doing so will invalidate the warranty on the unit and may cause unnecessary damage. The unit contains no user-serviceable parts.
- Only use fresh batteries as specified in the user manual. Do not mix new and old batteries.
- Due to printing limitations, the displays shown in this manual may differ from the actual display.
- The contents of this manual may not be reproduced without the permission of the manufacturer.

NOTE The technical specifications for this product and the contents of the user manual are subject to change without notice.

SPECIFICATIONS

TYPE	DESCRIPTION
MAIN UNIT	
Display rainfall range (total)	0 to 25.4 m (0 to 999.99 in)
Display rainfall range (history / daily)	0 to 2.54 m (0 to 99.99 in)
Rainfall resolution	1 mm (0.04 in)
Measuring accuracy	0 -15 mm per hour: +/- 10% Over 15 mm per hour: +/- 15%
Display temperature range	-5°C to 50°C (23°F to 122°F)
Operating temperature range	-5°C to 50°C (23°F to 122°F)
REMOTE RAIN COLLECTOR (PCR122)	
RF transmission frequency	433 MHz
RF transmission protocol	2.1
RF transmission range	30 m (100 ft)
Operating temperature range	1°C to 60°C (34°F to 140°F)
ALARM CLOCK AND CALENDAR	
Clock	HH:MM 12-hour format
Calendar	Month / Day, Day / Month
Alarm	1-min. daily alarm
POWER	
Main unit	2 UM-4 or "AAA" 1.5V alkaline batteries
Rain collector	2 UM-3 or "AA" 1.5V alkaline batteries
WEIGHT	
Main unit	134 g (4.71 lbs)
Remote rain collector	9.2 oz (260 g)
DIMENSIONS	
Main Unit L x W x H	107x 87 x 56 mm (4.2 x 3.4 x 2.2 in)
Remote rain collector Diam x H	140 x 145 mm (5.5 x 5.7 in)

NOTE It is recommended that you use alkaline batteries with this product for longer performance.

ABOUT OREGON SCIENTIFIC

Visit our website (www.oregonscientific.com) to learn more about Oregon Scientific products such as digital cameras; MP3 players; children's electronic learning products and games; projection clocks; health and fitness gear; weather stations; and digital and conference phones. The website also includes contact information for our Customer Care department in case you need to reach us, as well as frequently asked questions and customer downloads.

We hope you will find all the information you need on our website, however if you're in the US and would like to contact the Oregon Scientific Customer Care department directly, please visit:
www2.oregonscientific.com/service/default.asp
OR
call 1-800-853-8883.
For international inquiries, please visit:
www2.oregonscientific.com/about/International.asp

FCC STATEMENT

This device complies with Part 15 of the FCC Rules. Operation is subject to the following two conditions: (1) This device may not cause harmful interference, and (2) This device must accept any interference received, including interference that may cause undesired operation.

WARNING Changes or modifications not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

NOTE This equipment has been tested and found to comply with the limits for a Class B digital device, pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference in a residential installation.

This equipment generates, uses and can radiate radio frequency energy and, if not installed and used in accordance with the instructions, may cause harmful interference to radio communications. However, there is no guarantee that interference will not occur in a particular installation. If this equipment does cause harmful interference to radio or television reception, which can be determined by turning the equipment off and on, the user is encouraged to try to correct the interference by one or more of the following measures:

- Reorient or relocate the receiving antenna.
- Increase the separation between the equipment and receiver.
- Connect the equipment into an outlet on a circuit different from that to which the receiver is connected.
- Consult the dealer or an experienced radio / TV technician for help.

DECLARATION OF CONFORMITY

The following information is not to be used as contact for support or sales. Please call our customer service number (listed on our website at www.oregonscientific.com), or on the warranty card for this product) for all inquiries instead.

We

Name: Oregon Scientific, Inc.
Address: 19861 SW 95th Ave., Tualatin, Oregon 97062 USA
Telephone No.: 1-800-853-8883

declare that the product

Product No.: RGR682
Product Name: Wireless Rain Gauge with 10-Day Rainfall Memory and Digital Clock
Manufacturer: IDT Technology Limited
Address: Block C, 9/F, Kaiser Estate, Phase 1,41 Man Yue St., Hung Hom, Kowloon, Hong Kong

is in conformity with Part 15 of the FCC Rules. Operation is subject to the following two conditions: 1) This device may not cause harmful interference. 2) This device must accept any interference received, including interference that may cause undesired operation.

086L004267-054

Appendix C.
Instruction Sheets Provided to Volunteer Rainfall Monitors

Rainfall Monitoring:
Upper Fish River Source Tracking Project
Contact: Michael Shelton, Weeks Bay Reserve 251-331-1703 or michael.shelton@dcnr.alabama.gov

What is the value in my rainfall monitoring?

The goal of the source tracking project is to identify the sources of pathogenic bacteria in the Fish River. These bacteria increase in the water when it rains due to runoff from the landscape. Since rainfall in our area can be isolated, rainfall data need to be collected in several locations along Fish River to insure that water samples may be collected in response to the rain events. You help and the rainfall number you collect will help me collect samples early in rain events when the bacteria counts are likely highest. You will help us get an accurate accounting of rainfall, bacteria numbers and potential sources when the bacteria are further tested.

How to use your electronic rainfall monitor?

The monitor basically runs itself with no outside intervention. The unit is self-tipping and does not need to be emptied. Total and daily rainfall is collected. The daily rainfall resets to zero at midnight on each evening, so daily rainfall is collected from midnight to midnight as a 24 hour cycle. The total rainfall is collected over time providing a long-term assessment of rain amounts over the monitoring period.

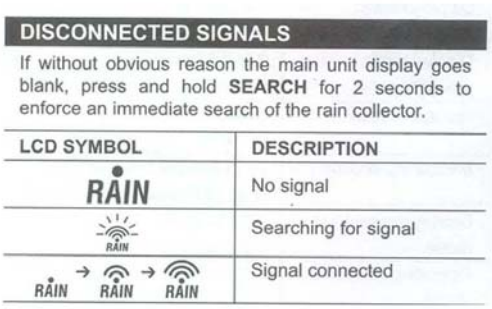
As a volunteer monitor, what do I have to do to the electronic rainfall monitor?

You have been provided a monthly rainfall data sheet, a rainfall bucket unit posted outside your home and a receiver unit for inside your home. Since the electronic monitor does just about everything for you, there is nothing for you to do to the bucket unit or receiver unit on a regular basis. No buttons to push or anything.

Once installed outside your home, please do not bump it with the mower or anything else. The outside unit needs to remain level to function correctly. If the rainfall monitor is not collecting rainfall amounts or is doing anything you cannot explain, please call Mike Shelton at 251-331-1703 or email: michael.shelton@dcnr.alabama.gov.

What do I do if the rainfall monitor has a problem?

Because the outside and inside units are designed for easy use, there are very few things that can go wrong. One is the battery. Please keep an eye on the low battery warning signal which is in the lower right corner of the display on the indoor receiver unit above the seconds register on your clock. If you see the low battery warning signal, please call Mike Shelton at 251-331-1703 or email: michael.shelton@dcnr.alabama.gov. Also, keep an eye on the connection signals. These signals tell you if the outside unit is talking to the indoor receiver unit. There are only 3 signals.



DISCONNECTED SIGNALS

If without obvious reason the main unit display goes blank, press and hold **SEARCH** for 2 seconds to enforce an immediate search of the rain collector.

LCD SYMBOL	DESCRIPTION
RAIN	No signal
RAIN	Searching for signal
RAIN → RAIN → RAIN	Signal connected

If you just see the word RAIN with a dot over the word, there is a problem. Press the SEARCH button on back of the indoor receiver unit one time. The indoor receiver unit will search until the connection is restored. The signal showing connection is the bottom signal on the figure. Over RAIN will be arcs that are increase to 3 then go back to none. That indicates a good signal. If you have a connections problem that cannot be fixed by pressing the SEARCH button, please call Mike Shelton at 251-331-1703 or email: michael.shelton@dcnr.alabama.gov.

Again, if the rainfall monitor is not collecting rainfall amounts or is doing anything you cannot explain, please call Mike Shelton at 251-331-1703 or email: michael.shelton@dcnr.alabama.gov.

What rainfall data do I record?

You have been provided several monthly data sheets, one for each month. There are 3 columns on the data sheet. The first column is filled in with the dates. Rainfall data are reported on the display on the indoor receiver unit. The top number on the display is the TOTAL rainfall. The middle number on the display is the DAILY rainfall. Please record the TOTAL rainfall and the DAILY rainfall in the appropriate column.

Please check your rainfall monitor and record data at 1900 hours or 7:00pm each day or as close to that time as is practicable. If you are out of town or otherwise unavailable, please resume reading the indoor receiver unit when you return.

How much rainfall triggers a water sampling event?

Whenever you record a **DAILY total of 0.1 inches or greater**, please give me a call at 251-331-1703 or email: michael.shelton@dcnr.alabama.gov. You are free to contact me in the evenings around 7:00pm or 1900 hr when you take your reading of TOTAL rainfall and DAILY rainfall. I will take a water sample in response to your contact, either that evening or early the following morning.

Appendix D.
Mueller-Hinton Media Specifications, Becton-Dickinson (Franklin Lakes, NJ)

BD BBL™ Prepared Medium for the Cultivation of Microorganisms

Mueller Hinton Broth

CE 8806691JAA 2003/08

See symbol glossary at end of insert. / Se symbolglossaret i slutningen af indlægssedlen. / Voir le glossaire des symboles à la fin de la notice. / Siehe Symbol-Erklärungen am Ende der Packungsbeilage. / Δείτε το γλωσσάριο των συμβόλων στο τέλος του ένθετου. / Vedere il glossario dei simboli alla fine del foglio illustrativo. / Consulte o glossário de símbolos no fim do folheto informativo. / Consulte el glosario de símbolos al final del prospecto. / Se symbolförteckningen vid slutet av bipacksedeln.

INTENDED USE

Mueller Hinton Broth is a general-purpose medium that may be used in the cultivation of a wide variety of fastidious and nonfastidious microorganisms. This formulation has not had its calcium and magnesium ion concentrations adjusted to make it suitable for use in quantitative procedures for antimicrobial susceptibility testing.

SUMMARY AND EXPLANATION

The Mueller Hinton formulation was originally developed as a simple, transparent agar medium for the cultivation of pathogenic *Neisseria*.[1] Other media were developed that replaced the use of Mueller Hinton Agar for the cultivation of pathogenic *Neisseria*, but it became widely used in the determination of sulfonamide resistance of gonococci and other organisms. It now is recommended as the test medium for use in antimicrobial susceptibility testing.[2,3]

Mueller Hinton Broth, unadjusted, has a formula similar to that of the solid medium, but without agar, for use when fluid medium is preferred. It may be used for the general cultivation of bacteria.

PRINCIPLES OF THE PROCEDURE

Acid hydrolysate of casein and beef extract supply amino acids and other nitrogenous substances, minerals, some vitamins and other nutrients to support the growth of microorganisms. Starch acts as a protective colloid against toxic substances that may be present in the medium. Hydrolysis of the starch during autoclaving provides a small amount of dextrose, which is a source of energy.

REAGENTS

Mueller Hinton Broth

Approximate Formula* Per Liter Purified Water

Acid Hydrolysate of Casein	17.5 g
Beef Extract	3.0 g
Starch	1.5 g

*Adjusted and/or supplemented as required to meet performance criteria.

Warnings and Precautions:

For *in vitro* Diagnostic Use.

Tubes and bottles with tight caps should be opened carefully to avoid injury due to breakage of glass.

Observe aseptic techniques and established precautions against microbiological hazards throughout all procedures. After use, prepared tubes, specimen containers and other contaminated materials must be sterilized by autoclaving before discarding.

Storage Instructions: On receipt, store tubes and bottles in the dark at 2 to 25°C. Avoid freezing and overheating. Do not open until ready to use. Minimize exposure to light. Tubed and bottled media stored as labeled until just prior to use may be inoculated up to the expiration date and incubated for the recommended incubation times. Allow the medium to warm to room temperature before inoculation.

Product Deterioration: Do not use tubes or bottles if they show evidence of microbial contamination, evaporation, precipitation or other signs of deterioration.

SPECIMEN COLLECTION AND HANDLING

This medium is not suitable for use directly with specimens or other materials containing mixed microbial flora except as a "backup" enrichment broth in addition to primary plating media. Consult appropriate references for further information.[4-6]

PROCEDURE

Material Provided: Mueller Hinton Broth

Materials Required But Not Provided: Ancillary culture media, reagents, quality control organisms and laboratory equipment as required for this procedure.

Test Procedure: Observe aseptic techniques and established precautions against microbiological hazards throughout all procedures.

Organisms to be subcultured must first be isolated in pure culture on an appropriate solid medium. Transfer growth from the isolation medium to Mueller Hinton Broth using standard bacteriologic techniques.[5-7]

For enrichment purposes, inoculate the specimen onto primary media and then into the broth according to recommended procedures.

Incubate tubes and bottles at 35°C under conditions appropriate for the organism being cultured.

User Quality Control:

1. Examine the tubes and bottles for signs of deterioration as described under "Product Deterioration".
2. Check performance by inoculating a representative sample of tubes and bottles with pure cultures of stable control organisms that give known, desired reactions. The following test strains are recommended:

Contact your local BD representative for instructions. / Veuillez contacter le Service d'Assistance Technique de BD pour toute instruction. / Um Anleitungen zu erhalten, wenden Sie sich bitte an Ihren BD-Kundendienst. / Contattare il rappresentante BD di zona per ottenere il foglietto illustrativo. / Para obtener el prospecto del producto, comuníquese con el representante de BD.

TEST STRAIN	EXPECTED RESULTS
Escherichia coli ATCC™ 25922	Growth
Staphylococcus aureus ATCC 25923	Growth
Enterococcus faecalis ATCC 29212	Growth

Quality control requirements must be performed in accordance with applicable local, state and/or federal regulations or accreditation requirements and your laboratory's standard Quality Control procedures. It is recommended that the user refer to pertinent NCCLS guidance and CLIA regulations for appropriate Quality Control practices.

RESULTS

Growth in broth media is indicated by the presence of turbidity compared with an uninoculated control.

LIMITATIONS OF THE PROCEDURE

Enrichment broths should not be used as the sole isolation medium. They are to be used in conjunction with selective and nonselective plating media to increase the probability of isolating pathogens, especially when they may be present in small numbers.

For identification, organisms must be in pure culture. Morphological, biochemical and/or serological tests should be performed for final identification. Consult appropriate texts for further information.[4-7]

PERFORMANCE CHARACTERISTICS

Prior to release, all lots of Mueller Hinton Broth are tested for performance characteristics. Representative samples of the lot are tested with cell suspensions of *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, diluted in normal saline to yield 10^3 to 10^4 CFU per tube of broth. Tubes are incubated with loose caps at 35 ± 2°C for one day in an aerobic atmosphere. Growth is observed with all organisms.

AVAILABILITY

Cat. No.	Description
296195	BBL™ Mueller Hinton Broth, 2 mL, Pkg. of 10 size K tubes
296164	BBL™ Mueller Hinton Broth, 2 mL, Ctn. of 100 size K tubes
297220	BBL™ Mueller Hinton Broth, 5 mL, Pkg. of 10 size C tubes
295834	BBL™ Mueller Hinton Broth, 5 mL, Ctn. of 100 size C tubes
297868	BBL™ Mueller Hinton Broth, 100 mL, bottle

REFERENCES

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Manufacturer / Producent / Fabrikant / Valmistaja / Fabricant / Hersteller / Κατασκευαστής / Ditta produttrice / Fabrikant / Fabricante / Tillverkare

Use by / Anvendes før / Houdbaar tot / Viimeinkäyttöpäivä / A utiliser avant / Verwendbar bis / Ημερομηνία λήξης / Usare entro / Brukes før / Utilizar em / Usar antes de / Använd före / YYYY-MM-DD / YYYY-MM (MM = end of month) / ÅÅÅÅ-MM-DD / ÅÅÅÅ-MM (MM = slutning af måned) / JJJJ-MM-DD / JJJJ-MM (MM = einde maand) VVVV-KK-PP / VVVV-KK (kuukauden loppuun mennessä) AAAA-MM-JJ / AAAA-MM (MM = fin du mois) / JJJJ-MM-TT / JJJJ-MM (MM = Monatsende) / EEEE-MM-HH / EEEE-MM (MM = τέλος του μήνα) / AAAA-MM-GG / AAAA-MM (MM = fine mese) / ÅÅÅÅ-MM-DD / ÅÅÅÅ-MM (MM = slutten av måneden) AAAA-MM-DD / AAAA-MM (MM = fim do mês) / aaaa-mm-dd / aaaa-mm (mm = fin del mes) / ÅÅÅÅ-MM-DD / ÅÅÅÅ-MM (MM = slutet på månaden)

REF Catalog number / Katalognummer / Katalogusnummer / Tuotenumero / Numéro catalogue / Bestellnummer / Αριθμός καταλόγου / Numero di catalogo / Katalognummer / Número do catálogo / Número de catálogo / Katalognummer

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IVD In Vitro Diagnostic Medical Device / In vitro diagnostisk medicinsk anordning / Medisch hulpmiddel voor in vitro diagnose / Lääkinnällinen in vitro -diagnostiikkalaite / Dispositif médical de diagnostic in vitro / Medizinisches In-vitro-Diagnostikum / In vitro διαγνωστική ιατρική συσκευή / Dispositivo medico diagnostico in vitro. / In vitro diagnostisk medisinsk utstyr / Dispositivo médico para diagnóstico in vitro / Dispositivo médico de diagnóstico in vitro / Medicinsk anordning för in vitro-diagnostik

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Appendix F

Media Coverage of Upper Fish River Project

SEEKING THE SOURCE

Study looking for origins of Fish River pollution

By RYAN DEZEMBER
Staff Reporter

In an attempt to figure out how exactly bacterial pollutants are entering Fish River, state and federal scientists have begun a two-year study in which they hope to determine the source of contaminants — be they human, domesticated animals or wildlife — at various points along the waterway.

To do so, they'll use a process called antibiotic resistance analysis, said Michael Shelton, watershed coordinator at Weeks Bay National Estuarine Research Reserve.

Bacteria develop resistance to antibiotics over time. And because the antibiotics taken by humans are different from those given to domesticated animals — and wildlife generally aren't exposed to antibiotics — scientists believe they'll be able to pinpoint where the pollutants in each water sample originated, based on the resistance each sample shows to various antibodies, Shelton said.

The study will focus on E. coli, bacteria commonly found in the lower intestines of warm-blooded animals, Shelton said.

"The ultimate goal is to identify the sources of the pathogen contamination," Shelton said. "Once you identify the sources with reasonable certainty, you can make better management decisions."

The study will work like this: Shelton and a team of local volunteers will collect samples after rainstorms from about six points along Fish River between Interstate 10 and its confluence with Polecat Creek north of Marlow. Those water samples will be taken back to a lab at the Weeks Bay Reserve where scientists will grow the bacteria found in each sample.

Plates of the bacteria will then be shipped to Brian Burnes, an associate professor of biology at the University of West Alabama, who will apply various antibiotics to the samples to determine whether the pollutants have come from humans, wildlife or domestic animals, Shelton said.

Please see *Study* Page 7 ▶

RYAN DEZEMBER/Register

Scientists are embarking on a two-year study aimed at pinpointing how bacterial pollutants are entering Fish River. The study will use antibiotic resistance analysis to determine whether the bacteria originate in humans, domesticated animals or wildlife.

Study seeks sources of pollution

▸ Continued from Page 1

Though Fish River's water quality is "reasonably good," the river has had past problems with various pathogens as well as mercury contamination, Shelton said. Originating near the Stapleton community, Fish River is met by numerous tributaries before ending in Weeks Bay west of Foley.

The Southern Environmental Law Center has named Weeks Bay as one of the 10 most endangered places in the South. Addressing pathogen contamination in the waters flowing into the bay is one of the priorities outlined in the its watershed management plan, Shelton said in a news release.

To help with the study, scientists are looking for volunteers who live on or near the river. Shelton said volunteers who can monitor rainfall are the most crucial.

Volunteers will be given rain gauges, supplies and training and they'll alert Shelton when there is a storm that dumps at least a half-inch of rain preceded by at least three days of dry weather. If they're able, Shelton said the volunteers would also be trained to collect samples from the river after these rain events. If not, he said, the scientists involved in the study will retrieve a sample.

"Volunteers can make a great contribution to the success of the project," Shelton said.

Those interested in participating can call Shelton at the Weeks Bay Reserve at 251-928-9792.

BaldwinCountyNOW.com
A Gulf Coast Information Source for South Alabama

Print Page

Source testing for Fish River pollutants planned

Volunteers needed to help with environmental effort

By Curt Chapman
Staff Writer

(Created: Monday, February 16, 2009 10:05 AM CST)

FAIRHOPE, Ala. — It's no secret that contaminants continue to plague Fish River, as well as other coastal freshwater rivers and streams. Sediment, nutrients and mercury contribute to the problem, but bacteria is among the greatest concerns officials have.

Fecal material from humans, household pets, domestic livestock and wildlife finds it way into the water, often affecting seafood and sickening people who consume it. Swimming in water contaminated by fecal bacteria can cause skin problems, and even more severe illnesses if the water is ingested.

Chances are you've seen the Alabama Department of Public Health's color-coded beach monitoring signs that indicate the bacterial levels in the water at more than two dozen beaches on both sides of Mobile Bay. The question is where do these bacterial pollutants come from, and how can they be stopped?

A new study could come up with an answer, thanks to the combined efforts of the Weeks Bay Foundation (WBF); Weeks Bay Reserve (WBR) — a partnership between the Alabama Department of Conservation and Natural Resources State Lands Division and the National Estuarine Research Reserve System of the National Oceanic and Atmospheric Administration — Mobile Bay National Estuary Program (MBNEP), the University of West Alabama (UWA) and the Weeks Bay Watershed Project (WBWP).

The agencies initiated a point source pollution research program to identify the origin of bacteria found in the waterway. MBNEP and the U.S. Environmental Protection Agency identified the upper river as a priority area for projects that address water pollution, and as a result provided grant money to conduct the research.

Mike Shelton, WBR watershed coordinator and one of the research leaders, said, "What we're doing right now is looking at an important area of the river identified as a concern by MBNEP. It's a manageable size of the watershed. We're going to develop techniques and a pathogen management plan we can develop for other parts of the river."

Fish River is included on the Clean Water Act list of impaired water as contaminated with pathogen bacteria. The river is one of the two main tributaries to Weeks Bay, designated as an Outstanding National Resource Water, and it is one of the 10 Most Endangered Places in the South as reported last month by the Southern Environmental Law Center.

The research project will be carried out with help from a scientist and student from UWA and volunteers.

Dr. Brian Burnes of UWA will perform a portion of the identification using antibiotic resistance analysis. The process examines the way E. coli from the water grows or does not grow in the presence of different kinds of antibiotic. Much has been reported of the resistance to antibiotics potentially harmful bacteria can develop. The source tracking method relies on antibiotic resistance to tell about a bacterium's source.

"Because humans are prescribed certain kinds of antibiotics, E. coli are going to develop resistance to that level," Shelton said. "Even wildlife will exhibit some type of resistance to some doses and some types of antibiotics. We'll compare the resistance to known bacteria and determine with statistical massaging the bacteria came from a specific source."

Bacteria have the ability to develop resistance to antibiotics when exposed to the compounds over time. Bacteria in the digestive track of a human would have a certain resistance, for example, and those from different animals would have a different resistance pattern. Burnes will examine the resistance patterns from those known pathogen sources and compare them to bacteria from Fish River.

Shelton said when the source is identified, better management practices can be applied to the problem and reduce or eliminate the pollution. He is coordinating water sample collection.

Sample sites are located from Fish River's confluence with Polecat Creek north to Interstate 10. The sampling will occur at seasonal increments at both normal flow in the river and under high flow conditions, such as after heavy rainfall.

Both the reserve and the foundation have long been involved in not only protecting the Weeks Bay watershed, but also in educating area residents about the ways to prevent water pollution in the first place.

As part of that ongoing effort, Shelton said volunteers are needed to host a rain gauge at their home and report heavy rain events, and if possible also go to one of the designated testing points on the river and take samples. Training, equipment and supplies will be provided.

"We would like them to monitor rainfall. A certain amount triggers a sampling event," he said. "The rainfall is going to flush whatever is on the ground. We want to find out what kind of things we see under baseflow conditions, and what we get when we have a high-water event."

Current fecal coliform monitoring conducted by Weeks Bay Water Watch includes locations spanning much of the accessible reaches of Fish River and several tributaries. Counts of bacteria in the upper river remain periodically high, but typically following rain events.

Shelton said, "Once you have reasonable certainty what the predominant source is for these cells, you develop strategies to address them. For domestic livestock, look at the landscape, work with these (ranchers) to get that fixed. We'll apply best management practices targeted at the sources."

Paul Dowsey, an experienced Weeks Bay Reserve volunteer, has been working with Shelton on the source tracking project since October. He collects rainfall data and E. coli samples when adequate rainfall occurs. As one might expect, data at the site near Marlow indicates that high levels of pathogens are present after significant rainfall events.

There is a great need to better identify sources so that better management programs may be developed to address the water quality problem, according to Shelton. Potential sources like pasture grazing remain, he noted, but with continuing development occurring in the upper Fish River watershed, additional sources like urban storm water runoff become more prevalent.

"There's a lot of potential sources out there, and we want to use our limited resources to address them, and not just shotgun them on the ground," Shelton added.

To volunteer for the point source pollution study, call Mike Shelton at (251) 928-9792, or e-mail him at michael.shelton@dcnr.alabama.gov.