Hancock Creek: Watershed Improvement Initiative

THE CITY OF WINCHESTER

Acknowledgments
This project relied on a number of individuals and organizations for its success. With sincere gratitude, the project would like to acknowledge the following individuals and agencies for their commitment and contributions to this project.

Winchester Municipal Utilities
Jon Walker
Doug Christopher
Kenny Book
Stephen Berry, CCGIS
Greg Madison
Jim Fettig, deceased
Randall Carrier, Clark County Health Department
Buddy Wilson, Clark County Health Department
Geoff Gearner, PhD. Morehead State University
Gary Epperson
Clare Sipple
Winchester Public Works
Marilyn Rowe
Debbie Bailey
Tammy Shelton
Joy Curtis
Ed Burtner
Ken Kerns
J.R. Wilhite
Brooke Shireman
Lajuanda Haight-Maybriar
Jim Roe
Barry Tonning
William & Lee Quisenberry
Rhonda Cromer
Clark County Elementary and Middle Schools
St. Agatha Academy
Kentucky Waterways Alliance
US EPA, Region IV
Daniel Bishop
CDP Engineers, Inc.
Bell Engineering, Inc.
Winchester-Clark County Planning Commission
Bluegrass Council Boy Scouts of America
Wilderness Road Girl Scouts
Morehead State University Biology Department

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Executive Summary

The Hancock Creek: Watershed Improvement Initiative funded by the Kentucky Division of Water (DOW) through a 319(h) Nonpoint Source Pollution Control Grant and through matching efforts by the City of Winchester (COW), Winchester Municipal Utilities, and the Clark County Fiscal Court, was designed to implement the Hancock Creek Watershed Plan (HCWP) [7] and improve water quality conditions in the Hancock Creek watershed located in Clark County, Kentucky. Although not the lead on the project, the Strodes Creek Conservancy (SCC), an active watershed stakeholder group, helped to implement the grant. The City of Winchester agreed to serve as the lead on the grant by:

a) Conducting a thorough investigation of stream water quality through microbial source tracking (MST) technology to identify the source of any pollutants and
b) Developing and offering to the public best management practices (BMPs) that could reduce/eliminate the pollutants.

Land use within the Hancock Creek watershed is quite diverse and has the potential for substantial development in the near future. Current, potential sources of pollution come from several sources – residential and business septic tanks, residential and business package plants, runoff from agricultural operations, permitted industrial discharges, and runoff from a major transportation corridor, Interstate 64. Future sources of pollution include runoff from impervious surfaces, nonpoint source pollution from lawn care and maintenance, and construction sedimentation as the approximately 9,361 housing units that have been approved for development in the watershed are constructed.

To identify current sources of bacterial contamination within the watershed, the SCC used MST of water samples collected throughout the watershed. This tracking identified the presence of both cattle and human contamination markers in the samples. BMPs for both agricultural and residential use were then developed. Implementation of agricultural BMPs in the watershed met with limited success primarily due to farmers' reluctance to change established, profitable land use cattle operations. Residential BMPs (primarily repair and maintenance of septic systems) met with much greater acceptance and success.

To address future, potential development, the SCC developed (via contract with an engineering firm) recommended changes to the county's subdivision regulations that would allow developers the opportunity to use low impact development (LID) practices. LID is an approach to land development that uses the natural movement of water within an ecosystem to reduce the impact of development on water quality. The Winchester/Clark County Planning Commission has agreed to implement these practices in their subdivision regulations.

The SCC also increased the public's awareness of the watershed's water quality through educational outreaches, restoration projects, onsite wastewater projects, and establishment of vegetated riparian zones. The BMPS, regulatory changes, and educational outreaches accomplished by this grant not only resulted in immediate improvement to the water quality in Hancock Creek but also provided the framework for future generations to preserve and protect the stream for years to come.

Introduction & Background

Hancock Creek joins Strodes Creek just north of Winchester, a stream known in the past for black bass and good water quality. However, Strodes Creek today is listed by Kentucky Division of Water (KDOW) as impaired for primary contact recreation, and only partially supports aquatic life. Causes of impairment for Strodes Creek include pathogens, nutrients, siltation, and organic enrichment. The Hancock Creek watershed is quite diverse and has potential for significant change in the future. The watershed has the potential to receive pollution from such sources as septic tanks, package plants, agricultural land, subdivision development, commercial and industrial businesses, sewage lift stations, permitted industrial discharges and runoff from Interstate 64. Approximately 9,361 housing units have been approved for development in the future. Their inclusion to the landscape has the potential to impact water quality from the runoff from an increased number of impervious surfaces as well as nonpoint source pollution stemming from lawn care and maintenance, increased sedimentation from construction, and household pet waste to name a few.

In 2009, SCC began working with Kentucky Waterways Alliance (KWA) to develop a watershed plan for the Hancock Creek (HC) watershed as part of the review of the Watershed Planning Guidebook for Kentucky Communities. In June 2010, KDOW accepted the HCWP. However, as determined in the HCWP, additional work was needed to better determine pollution sources and conduct BMP implementation. This project sought funding to conduct MST to better determine pollution sources and implement the approved goals of the HCWP.

The SCC had a significant amount of fecal coliform, Escherichia coli (e-coli), and physical chemical data that showed stream impairment but was still missing a piece of the puzzle. To have a comprehensive plan, the SCC felt that it needed data that identify sources of the high levels of *E.coli* in the watershed. Identifying these sources would allow development and implementation of target BMPs. Four primary goals were identified for this project:

Goal 1: Conduct MST sampling to determine the origin of pollution sources.
Goal 2: Refine the current HCWP and submit to KDOW for acceptance.
Goal 3: Implement the identified BMP activities of the KDOW accepted HCWP.
Goal 4: Continue to build organizational strength to ensure that the SCC will exist beyond the timeline of the grant.

These goals were met by using the methods identified in the Materials & Methods section.

Materials & Methods

Hancock Creek, a tributary of Strodes Creek, is a main headwater stream of the South Fork of the Licking River. This stream is 7.65 miles long and has a drainage area of 12.9 square miles. The stream sits in an undulating, inner bluegrass landscape, over middle Ordovician age shale and limestone. The area was probably wooded, with some open areas (bluegrass savannah, canebrakes) before permanent human settlement.

Hancock Creek and its tributaries lie in the northwestern section of Clark County, which is a 210-year-old community. The watershed is in an ever-growing area of the county but still has agriculture as its predominant land use (See Figure 1). Development over the past 50 years includes three mobile home parks, five residential subdivisions, one crossroads community, three light industrial areas, and five

general business/professional office developments. Approximately 9,361 housing units have been approved for future development.

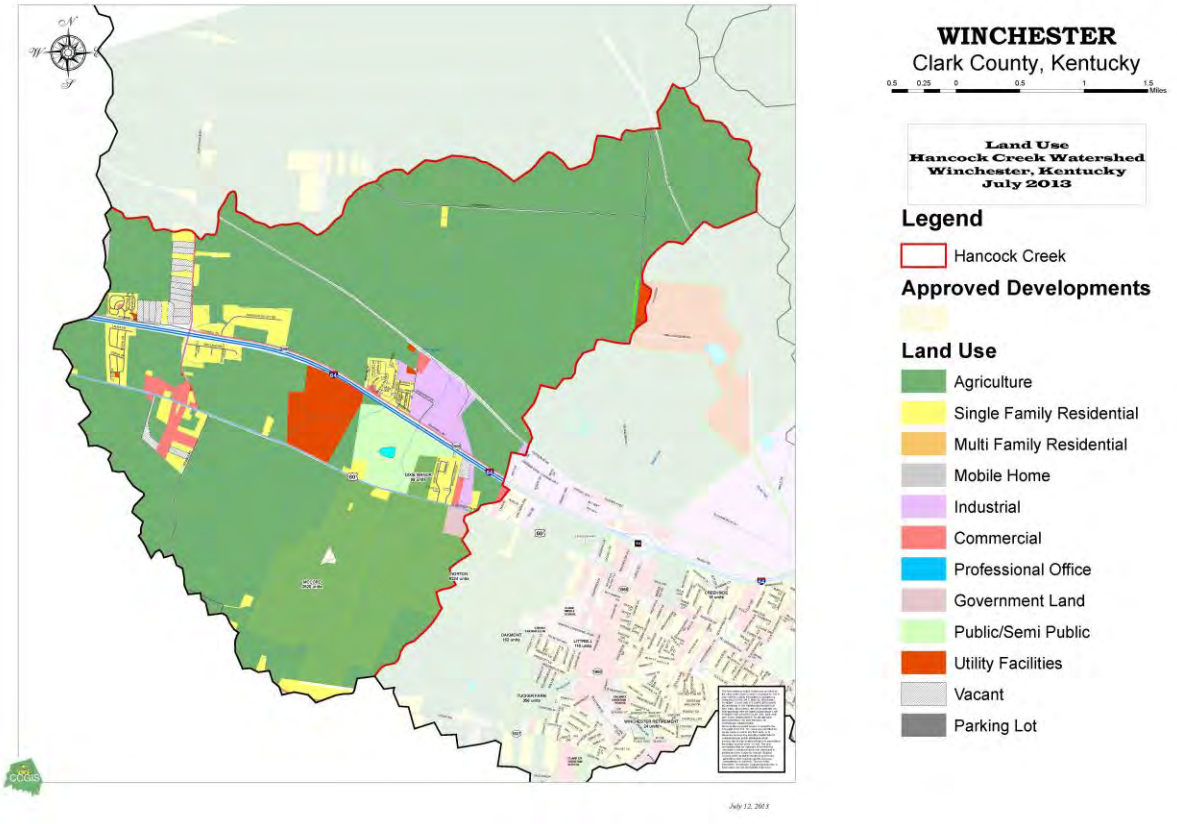

Figure 1. Hancock Creek Watershed Map

While planning this project, it was discovered that significant amounts of data existed for the Hancock Creek watershed. Through previous watershed planning development efforts, it was determined that additional sampling locations and collection of additional parameters were needed to allow for better source determination and targeting of BMPs. This project included the review of existing water quality data and analysis in the original HCWP to determine where additional water quality data was needed. The watershed plan development team determined that additional sampling stations and the use of MST as a way to determine if the Escherichia coliform (*E. coli*) bacteria was of human or animal (bovine) origin were needed.

All program efforts to improve water quality hinged on the results of the monitoring and MST results. The COW and SCC wanted to focus its resources on the sources that most adversely impacted the water quality in the streams. This section will first discuss the materials and methods developed for the monitoring and MST component of the grant and then focus on the programs that emerged as a result of the monitoring and MST data analysis.

Monitoring and Microbial Source Tracking

Bacteria are used as indicators of fecal contamination of waterways. Presently, E-coli, a bacterium associated with the feces of warm-blooded animals, is used as an indicator organism. Other microbial indicators include Enterococcus faecium and anaerobic bacteria of the Bacteroides- Prevotella group. SCC partnered with the Morehead State University Biology Department in testing water resources to identify bacteria contamination in the watershed and to determine if the bacterial source is from humans or livestock. Eight sites in the Hancock Creek Watershed were sampled in September and October 2011. The sites were thoughtfully selected to include sampling locations in the upper reaches of the watershed to a location just before Hancock Creek flows into Strodes Creek. Since the sampling was primarily conducted to help the SCC determine the sources of bacteria in the watershed, the sampling locations can be found below major residential subdivisions, agricultural areas, industrial activity, and a golf course. The Quality Assurance Project Plan (QAPP) required four sampling events to occur: one following each of two dry weather events (a period of seven days with a total rain accumulation of less than 0.1 inch), and two immediately following a wet weather event that proceeds a dry weather event. The summer and fall of 2011 were extremely dry with few rain events. The sampling team attempted their initial sample for the project for a dry event. However, conditions were too dry and several sites were ponded or had no flow. Since the sampling season was coming to an end, the sampling team collected a wet event sample on September 7, 2011 and followed up with a dry event sample on October 7, 2012. Because of this, the order in which the samples were to be taken was reversed and the project was able to conduct sampling and analyses for only one dry weather and one wet weather event. Therefore, a deviation from the original QAPP was requested from KDOW, which would omit two of the samples, one wet weather and one dry weather. The QAPP was approved on August 8, 2011, can be found in Appendix B.

Table 1. Sampling sites in the Hancock Creek Watershed.

HCW Sampling Site			
Site	Long	Lat	Description
HCW-1	-84.243927	+38.017175	B Golf Course
HCW-2	-84.283999	+38.029163	Verna Hill Package Plant
HCW-3	-84.244799	+38.027200	Wayland Drive
HCW-4	-84.278435	+38.036031	Yorktowne Package Plant
HCW-5	-84.259678	+38.033182	Culvert under Rockwell Road
HCW-6	-84.241263	+38.032636	Rockwell Package Plant
HCW-7	-84.236097	+38.029884	Southern States
HCW-8	-84.207441	+38.048460	Van Meter Road

Field activities conducted included the collection of bacteria samples, discharge measurements (flow), and physical chemical water quality data in wadeable streams. The bacteria samples collected included Escherichia or E. coli, human-specific E. faecium and Bacteriodes, and cattle-specific Bacteroides. The physical chemical data collected included dissolved oxygen, pH, conductivity, and temperature. The physical chemical data were collected using a YSI multiparameters probe. A turbidity measurement was not taken because the turbidity sensor was faulty at the time of sampling. A request to remove the turbidity measurement from the QAPP was approved by KDOW. The flow was measured with a SonTek/YSI FlowTracker®.

The MST process involved the use of polymerase chain reaction (PCR) to amplify DNA sequences that are unique to host-specific bacterial strains to a detectable level. PCR is a method used to amplify (increase copy number) specific sequences of DNA to detectable (observable) levels. Previous studies have identified bacterial strains that are uniquely present in human feces and cattle feces. Examples include human-specific strains of *Enterococcus faecium*, human-specific strains of *Bacteriodes*, and cattle-specific strains of *Bacteroides*. Published studies have also identified DNA sequences that are used to specifically identify these bacterial strains. We used PCR to look for the presence of host (human and/or cattle) specific *E. faecium* and *Bacteriodes* DNA sequences in water samples. The detection of those DNA sequences told us if human and/or cattle feces is present in sampled water.

In addition, the SCC wanted to determine the density of bacteria found at each of the sampling sites and prioritize BMP practices based on those findings. Bacterial densities (E. coli) were measured as per the Colilert method. For MST, the samplers were interested in molecular markers from other feces-associated bacteria; however, that approach was qualitative, and not quantitative. Both the E. coli data and the MST data provided information on the degree of fecal contamination present at a given sampling site, as well as the host source of that contamination. It was assumed that when analyzed together, those data would help determine the appropriate BMP to be implemented. Figure 2 shows the sampling locations for the project.

In addition to the aforementioned monitoring that took place during the project period, the SCC coordinated sampling events for Licking River Watershed Watch (LRWW). The Clark County LRWW team had three active samplers.

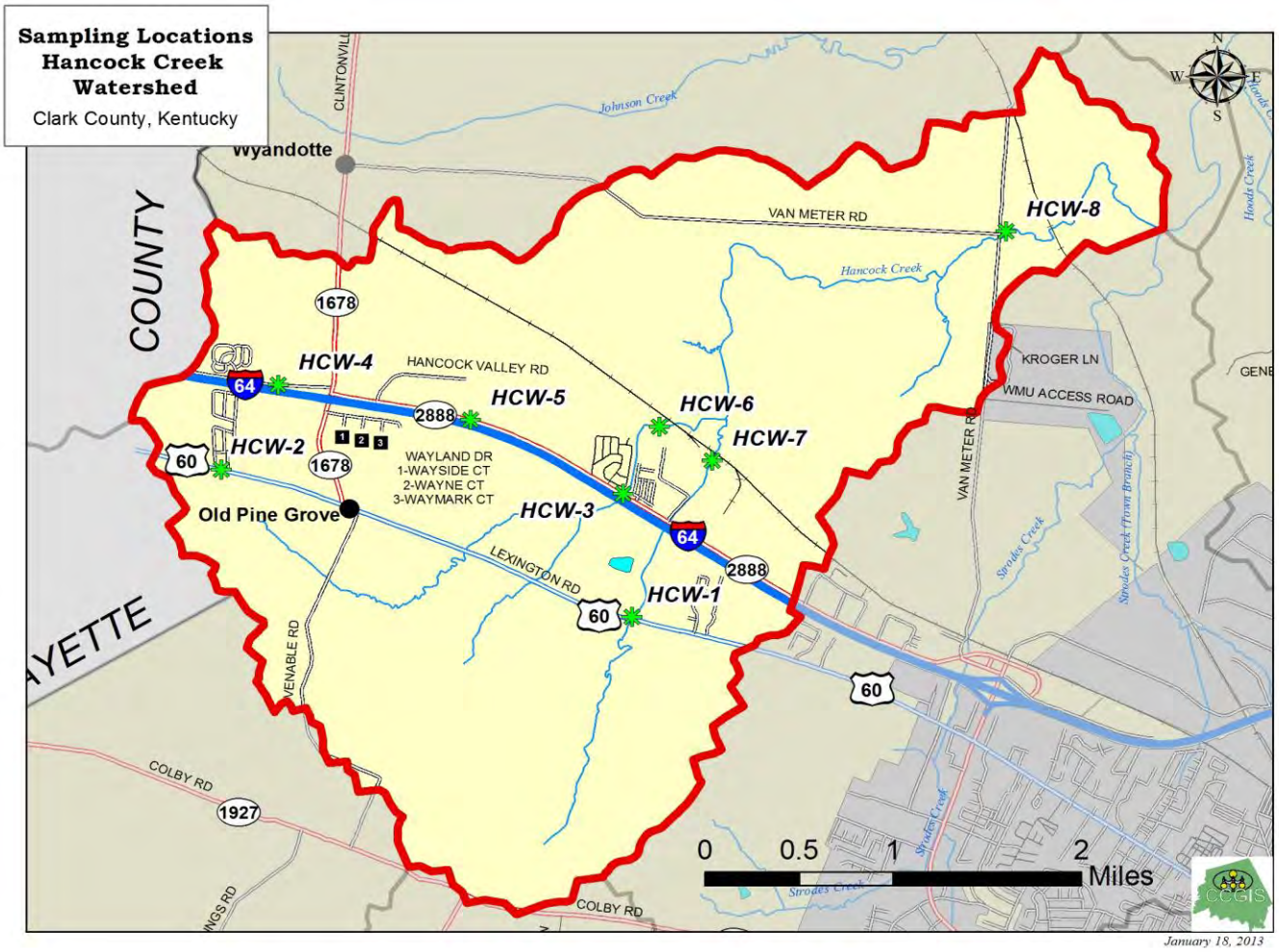

Figure 2. Hancock Creek Watershed Sampling Sites

Agriculture

The MST data analysis, along with the existing data from the previous HCWP, suggested that best management practices for the project should be targeted toward livestock operations. All of the large cattle producers in the watershed were personally contacted and offered best management practice (BMP) assistance. The project encouraged the funding and implementation of rotational grazing, stream exclusion fences, shade structures, and livestock alternative water supplies. A 20:80 cost share ratio was offered to help offset the cost of installing the BMPs with the landowner paying 20 percent of the cost of the practice and the SCC paying 80 percent. The 20 percent paid by the landowner could have been in the form of in-kind labor or tools.

Onsite Wastewater

The SCC, using a 2004 319(h) Nonpoint Source Pollution Control Grant (C9994861-04), worked diligently with homeowners to improve the efficiency and maintenance schedules of their home onsite wastewater systems. The inspections and tank pumping funded by that grant identified a number of failing systems. Many of these systems were installed before current environmental health standards and specifications were implemented. Water quality samples taken during this project indicated the presence of human bacteria markers. Specifically, a sampling location near Rockwell Road, in the vicinity of the Wayland Heights Subdivision, presented human bacteria markers that indicated

problems with septic systems in the area. As a result of the sampling data analysis and the system failures identified in the 2004 319(h) Nonpoint Source Pollution Control Grant (C9994861-04), onsite wastewater assistance was offered to homeowners that had expressed interest to the SCC in any funding opportunities to repair and/or replace their faulty systems. Once the funding became available, those homeowners were contacted by phone to discuss the potential projects. The homeowners were required to procure two bids from licensed onsite wastewater installers. The more competitive bid price was awarded the project. The homeowners were required to pay twenty percent (20%) of the total repair cost.

Tree Plantings

One simple best management practice to help improve water quality is planting trees along creek banks. Tree planting along creeks helps trap harmful nutrients present in water runoff, reduces erosion by slowing water flow, provides wildlife habitat, reduces in-stream temperatures to levels necessary for healthy aquatic life populations, and generally improves the overall aesthetic appeal of an area. Landowners in the watershed were previously contacted during the 2004 319(h) Nonpoint Source Pollution Control Grant (C9994861-04). As a continuation of those efforts, landowners that had previously participated in riparian planting projects were contacted during this grant period for permission to plant additional trees along Hancock Creek. Native trees were planted along the creek by Clark County students and the SCC project coordinator. The landowner was responsible for calling "Before You Dig" at 1 (800) 752-6007 to determine if underground lines were buried in the planting area. Landowners were required to water balled and burlaped trees during dry periods as necessary for at least the first year to ensure adequate hydration and survival.

Education

During the project period, the SCC implemented and maintained an active outreach program to the community. The outreach focused on water quality issues. Specifically, the SCC director spoke to approximately 30 elementary classrooms using the Enviroscape and lessons from the Project WET (Water Education for Teachers) curriculum. In addition, presentations were given to the Winchester Kiwanis Club, Winchester City Commission, Cub and Boy Scout troops, and Girl Scout troops. A newsletter was distributed to maintain contact with the constituency and make them aware of our MST project and findings as well as the funding opportunity for agricultural BMP implementation. The Strodes Creek Conservancy website was maintained and continues to educate viewers on basic water education, monitoring results, programs offered by the SCC, and a resource page. A stakeholder meeting to discuss the final version of the Hancock Creek Watershed Plan was conducted. The SCC also worked in partnership with the Licking River Watershed Watch to educate local citizens on effectively capturing quality-assured pathogen data.

As mentioned earlier, each individual participating in our onsite wastewater program was educated on the mechanics of a septic tank and what is necessary for the proper functioning of a septic system. A stream celebration, showcasing the Town Branch Stream Restoration Project (TBSRP), was held for scout troops. During this celebration, the designer of the TBSRP outlined the previous conditions of the degraded stream and the features of the restored stream. The troops learned that fully functioning streams can support a variety of aquatic life, hold water during peak flows and drought conditions, and cleanse the water it holds.

Stream Event

The Kentucky Transportation Cabinet (KYTC) partnered with SCC to restore approximately 7,000 feet of degraded Town Branch channel on City of Winchester property as an advanced stream mitigation site. KYTC incorporated several measures into the stream restoration project to improve stream water quality, in addition to restoring the physical stability and habitat of the stream. The SCC sponsored a day of celebration showcasing the Town Branch Stream Restoration Project (TBSRP). Approximately 80 boy and/or girl scouts and their chaperones visited the stream on October 27, 2013. The day started with a ride to the site by bus and disembarkation at a trail head. At the trail head, students engaged in a tree identification exercise before walking to the TBSRP site. There, the designer of the TBSRP discussed with the students the previous conditions of the degraded stream and the features of the restored stream. Visual aids were available to help the students understand the construction of the project and see a portion of the restoration that wasn't logistically available for viewing that day. After the TBSRP overview, scouts were assigned by scout level to a station. The following stations and curriculums were available to the scouts that day.

Station 1- Webelos

1. Learn to identify poisonous plants and venomous reptiles found in your area.
2. Give examples of:

A producer, a consumer, and a decomposer in the food chain of an ecosystem
One way humans have changed the balance of nature
How you can help protect the balance of nature

3. Identify a plant, bird, or wild animal that is found only in your area of the country. Tell why it survives only in your area.
4. Learn about aquatic ecosystems and wetlands in your area. Discuss with your Webelos den leader or activity badge counselor the important role aquatic ecosystems and wetlands play in supporting lifecycles of wildlife and humans.

Station 2-Bear Water & Soil Conservation Elective

1. Dig a hole or find an excavation project and describe the different layers of soil you see and feel.
2. Explore three kinds of earth by conducting a soil experiment.
3. What is erosion? Find out the kinds of grasses, trees, or ground cover you should plant in your area to help limit erosion.
4. Name four kinds of pollution.
5. Plant a tree.

Station 3-Wolf

1. Discuss ways that land, air, and water can get dirty.
2. Discuss three stories that tell how people are protecting our world.
3. Discuss ways you can save energy.
4. Discuss how recycling is done in Clark County and what items can be recycled.

A growing body of research indicates that direct exposure to nature is essential for healthy childhood development and for the physical and emotional health of children and adults.[1]
According to a study by Przybylski N. Weinstein and R. M. Ryan, nature makes you nicer, enhancing social interactions, value for community and close relationships.[2] By walking to our site, spending time outdoors, and including an educational component to the adventure, the stream event was a great success!

Low Impact Development (LID) Subdivision Regulations

The Hancock Creek Watershed team identified LID opportunities and ordinance assessment as two of their top four priorities for the community. With the help of the Winchester-Clark County Planning Commission (WCCPC) Director, the Center for Watershed Protection's Codes & Ordinances worksheet was completed to help the SCC determine if more flexible ordinances to allow for LID, green infrastructure, and stormwater friendly growth were available. Appendix C outlines the worksheet results. The results of the worksheet showed that developers did not have the option to develop in a low impact way in Clark County. With that knowledge in hand, the SCC hired an engineering firm to provide an overview of the principles of LID to the WCCPC. The commission seemed very receptive to the idea and agreed to conduct an internal review of the current subdivision regulations and identify the barriers to LID. A presentation of the findings was given to the Winchester City Commission, Clark County Fiscal Court, and the WCCPC. The WWCPC asked if the SCC could help in creating LID options in their subdivision regulations. This project paid to determine what options and recommendations were available to remove the barriers to LID. The project also paid for the drafts and final versions of the subdivision regulations that include an option for developers to build in a low impact way that reduces pervious pavement and encourages green infrastructure.

Hancock Creek Watershed Plan

The HCWP was developed as part of a 319(h) grant awarded to Kentucky Waterways Alliance. This grant built upon the work completed in that project. The SCC was interested in revising the HC WP to better determine the types of pollutants entering the creek as well as the sources of those pollutants. As mentioned previously, the SCC partnered with the Morehead State University Biology Department in testing water resources to identify bacteria contamination in the watershed and to determine if the bacterial source is from humans or livestock. In addition to the MST sampling, this project also installed some of the recommended BMPs that would improve water quality.

The HCWP has been updated to include:

1. The results of the MST monitoring,
2. A revised BMP Implementation Strategy, and
3. The load reductions expected as a result of the installation of BMPs.

The revised HCWP was submitted to KDOW for comment and acceptance.

Strodes Creek Conservancy Membership

The SCC has retained its charter members for nearly nine years. The SCC has asked program participants to join the board. Some have shown interest but have not actually attended the board meetings. The SCC newsletter reached out for more members by acknowledging that the current board is a committed group of individuals but that the SCC is always looking for more involvement from the people that actually live in the watershed. The board membership uses its voice to support water quality at community meetings and send letters in support of environmentally friendly projects to appropriate agencies, companies, and organizations. The SCC plans to maintain its presence in the community beyond the time frame of the grant and hopes to partner with the Winchester-Clark County Tree Board and Scout Troops to continue planting trees along streams in the Strodes Creek watershed.

Stormwater Treatment System

The SCC purchased a Fabco Stormsack stormwater treatment system, which removes pollutants from stormwater. The Fabco Stormsack unit maintains positive treatment of total suspended solids (TSS) year-round, regardless of flow rate. The unit is designed to remove a wide range of particle sizes (from 20 to 2,000 microns), as well as free oils, heavy metals and nutrients that attach to fine sediment. The SCC chose to purchase the unit to determine the amount of pollutants entering the stormwater system. In addition, its purchase may encourage the purchase of additional units in the MS4 area by the City of Winchester. The Fabco Stormsack was installed in a storm drain near 1 North Main Street.

Results & Discussion

The grant had a number of successes. This section will discuss the results of the project components.

Monitoring & Microbial Source Tracking
The SCC began this project with a considerable amount of water quality data in the Hancock Creek watershed. Even with the data that indicated high fecal coliform and e-coli levels, the question of the source of the pollutants still remained. The principle objective in this component of the study was to determine if fecal contamination in eight selected sampling sites of the Hancock Creek Watershed was of human and/or cattle origin. The full Hancock Creek Monitoring & Microbial Source Tracking Final Report can be found in Appendix D.

Results from the *E. coli* analysis are presented in Figures 3 and 4. Seven out of eight sampling sites assessed during the rain event in September 2011 exhibited *E. coli* counts that exceeded the KDOW primary contact recreation limit of 240 *E. coli* CFU/100 mL, whereas one out of the eight sites exhibited an *E. coli* count below the KDOW limit.[3] All eight of the sampling sites assessed during the dry event in October 2011 exhibited *E. coli* counts well below the KDOW limit. In fact, sites HCW-2, HCW-4, HCW-5, HCW-6, and HCW-7 had E. coli analyses results of 0 CFU/100 mL.

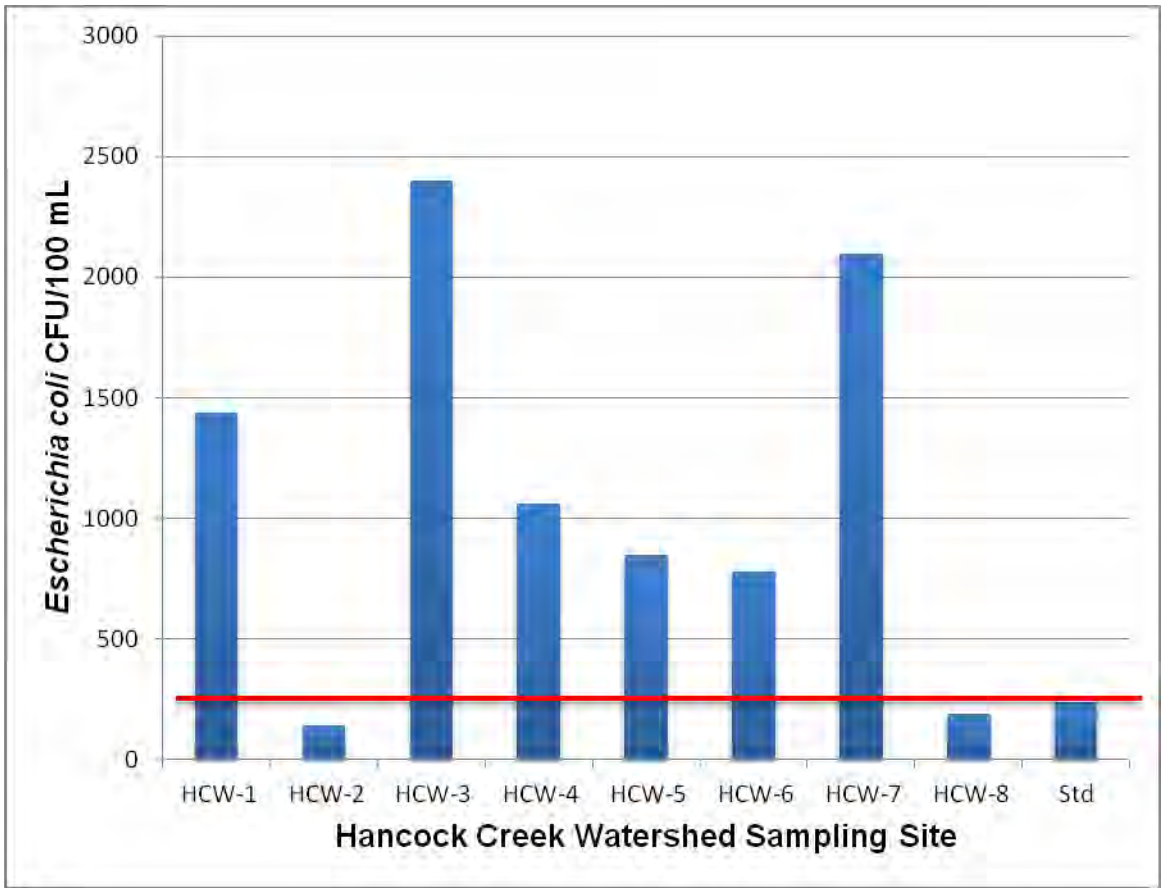

Figure 3. *Escherichia coli* counts in the Hancock Creek Watershed, 7 September 2011. The red line indicates the KDOW limit for primary contact reaction of 240 *E. coli* CFU/100 mL for single grab samples.[4]

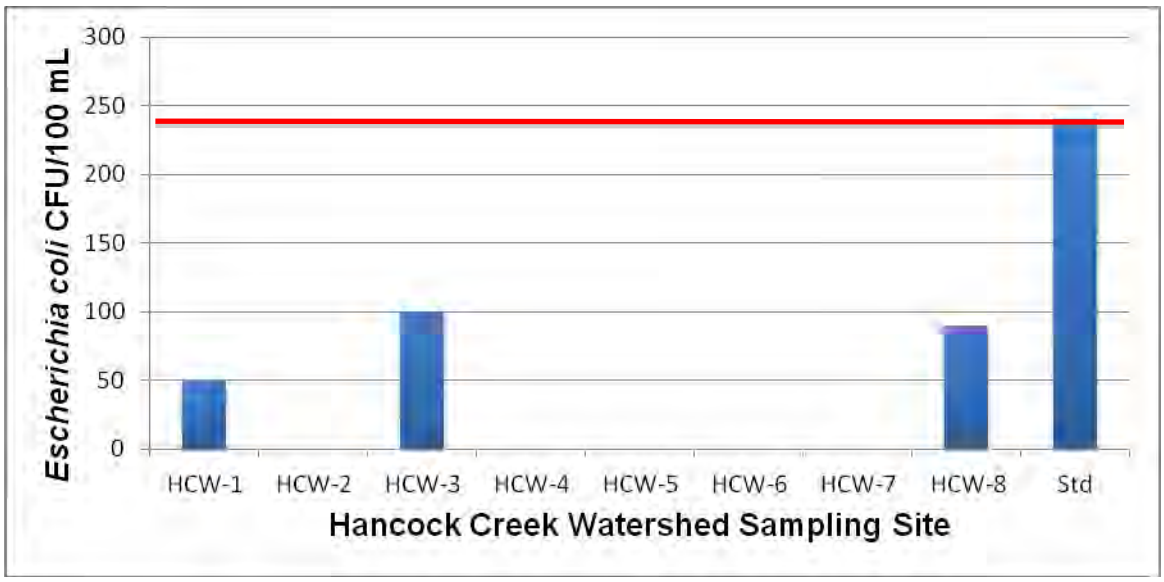

Figure 4. *Escherichia coli* counts in the Hancock Creek Watershed, 7 October 2011. The red line indicates the KDOW limit for primary contact reaction of 240 *E. coli* CFU/100 mL for single "grab" samples. Sites HCW-2, HCW-4, HCW-5, HCW-6, and HCW-7 had E. coli analyses results of 0 CFU/100 mL.[4]

Figure 5 shows a typical electrophoretic analysis of PCR products, while Table 2 summarizes the PCR results. Genetic markers for cattle-associated bacteria were present in all samples, except HCW-4, from the September sampling event; whereas human-associated bacterial genetic markers were found in the HCW-2 and HCW-5 samples. Cattle-associated bacterial genetic markers were present in six of eight samples collected during the October sampling event; while human-associated bacterial markers were present in one sample, HCW-4.

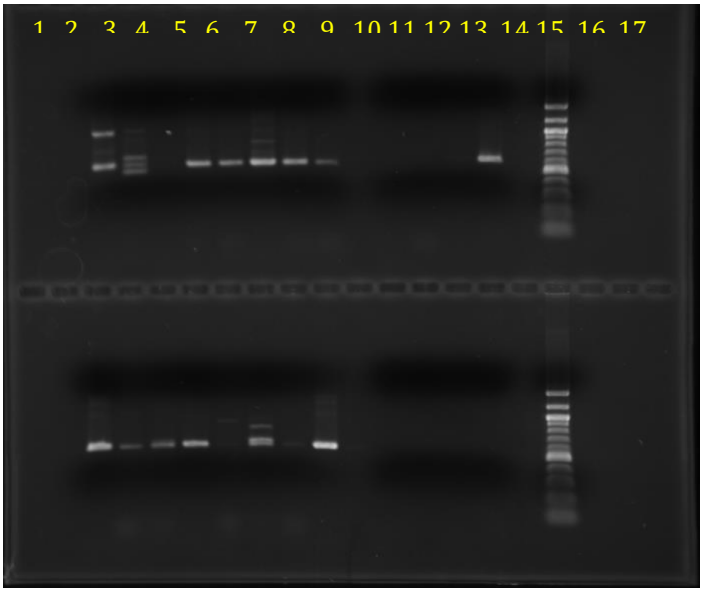

Figure 5. Ethidium bromide-stained 1.25% agarose gel of PCR products utilizing cattle-specific *Bacteroides* primers.

Table 2. Key to lane assignments in Figure 5 (above).

Top Row (7 Sept. Samples)		Bottom Row (12 Oct. Samples)	
Lane	**Sample**	**Lane**	**Sample**
1	none	1	none
2	none	2	none
3	HCW-1	3	HCW-1
4	HCW-2	4	HCW-2
5	HCW-4	5	HCW-4
6	HCW-5	6	HCW-5
7	HCW-6	7	HCW-6
8	HCW-7	8	HCW-7
9	HCW-3	9	HCW-3
10	HCW-8	10	HCW-8
11	none	11	none
12	HCW-8, no primers (- ctrl)	12	*E. coli* DNA (- ctrl)
13	primers only (- ctrl)	13	HCW-8, no primers (- ctrl)
14	pig feces DNA (- ctrl)	14	primers only (- ctrl)
15	cattle feces DNA (+ ctrl)	15	*E. coli* DNA (- ctrl)
16	none	16	none
17	100-bp DNA ladder	17	100-bp DNA ladder
18	none	18	none
19	none	19	none
20	none	20	none

Table 3. Summary of Polymerase Chain Reaction Analysis of DNA Extracted from Samples Collected in the Watershed. B/P refers to *Bacteroides-Provatella*; esp refers to enterococci.[5]

	7 September 2011			7 October 2011		
Sample Site	**Human B/P Marker***	**Cattle B/P Marker**	**Human esp Marker**	**Human B/P Marker**	**Cattle B/P Marker**	**Human esp Marker**
HCW-1	-	+	-	-	+	-
HCW-2	+	+	-	-	+	-
HCW-3	-	+	-	-	+	-
HCW-4	-	-	-	-	+	+
HCW-5	-	+	+	-	-	-
HCW-6	-	+	-	-	+	-
HCW-7	-	+	-	-	-	-
HCW-8	-	+	-	-	+	-

*The positive (+) sign indicates that the marker was present in the sample, while the negative (-) sign indicates that the marker was not present in the sample.

Table 4. Physical Chemical Results for Temperature, Specific Conductance, pH, and Dissolved Oxygen[3]

Sample ID	Collection Date	Collection Time	Temperature in °C	Conductivity mS/cm	pH	DO* mg/L
HCW-1	9/7/2011	11:34	17.16	0.54	8.01	7.33
HCW-2	9/7/2011	11:18	17.17	0.646	8.09	7.97
HCW-3	9/7/2011	11:48	17.14	0.56	7.94	6.73
HCW-4	9/7/2011	10:05	16.51	0.415	9.12	6.35
HCW-5	9/7/2011	10:33	17.75	0.679	8.28	5.84
HCW-6	9/7/2011	10:47	17.59	0.77	8.03	7.79
HCW-7	9/7/2011	11:03	17.69	0.763	8.01	7.39
HCW-8	9/7/2011	10:20	18.6	0.635	8.44	7.1
HCW-1	10/7/2011	No Data	No Data	No Data	No Data	No Data
HCW-2	10/7/2011	10:38	24.03	1.309	7.94	5.87
HCW-3	10/7/2011	No Data	No Data	No Data	No Data	No Data
HCW-4	10/7/2011	No Data	No Data	No Data	No Data	No Data
HCW-5	10/7/2011	No Data	No Data	No Data	No Data	No Data
HCW-6	10/7/2011	10:05	23.33	0.871	8.09	4.83
HCW-7	10/7/2011	9:53	22.41	0.746	8.31	4.69
HCW-8	10/7/2011	9:38	23.56	0.808	8.73	4.7

* DO indicates dissolved oxygen.

Please note that a turbidity measurement was not taken because the probe was broken. Also, data for the physical chemical data collected on October 7, 2011 is incomplete due to operator error.

The samples collected indicated that both cattle and humans are contributing to the bacteria levels in the streams. However, the cattle markers were more prevalent and indicated a higher level of contamination than that of human contamination. High E. coli counts after a rain event and low E. coli counts during a dry event indicate that rain water carried feces on the land surface into the watershed during the rain event. It should be noted that not all cattle operations contributed to high bacterial counts. For example, one of the sampling locations had cattle in the adjacent pasture and the cattle used the creek as a water source, but the samples taken were found to be within the KDOW limits for primary contact recreation in both of the samples taken.

Agriculture

Although BMP funding was available to landowners, the project did not have an agricultural BMP installation. Farmers were reluctant to install BMPs for a variety of reasons; primarily the uncertain economic benefit of replacing traditional practices with newer non-traditional practices. Other possible reasons for not participating in the project include the cost share payment associated with bmp installation and the loss of pasture when BMPS are installed.

Onsite Wastewater BMPs

The project helped install low pressure dosing systems at two homes in the Wayland Heights subdivision. Please see Figure 6 for the location of the installed systems. Both systems received new 1,500-gallon septic tanks, 1,000-gallon pump tanks, and low pressure piping in the drainfields. As a condition for participating in this project, homeowners were required to sign maintenance agreements. These agreements should prevent the future release of bacteria into the watershed. A copy of the maintenance agreement is in Appendix E.

A low-pressure dosing system treats wastewater and then pumps it into the soil several times daily. The system has three components: a series of tanks used to settle out and partially treat the wastewater; a pump tank for dosing wastewater to the distribution system; and a system for distributing the wastewater to the soil. The pump tank houses a pump that discharges wastewater to the distribution system three to four times a day. The distribution system consists of a small pipe with holes drilled in it, laid in narrow 6- to 12-inch-wide trenches. The pump discharges wastewater to the trenches. Once in the trench, the wastewater seeps into the soil. The soil provides most of the wastewater treatment. Soil particles filter solids and organic matter from the wastewater. Microbes in the soil break down the solids and kill the bacteria and pathogens in the wastewater.[6]

Tree Planting

During the project period, the SCC planted thirty (30) balled and burlaped trees and approximately 300 sapling trees in the Hancock Creek watershed. The letter offering the program to landowners can be found in Appendix F. Please see Figure 6 for tree planting locations.

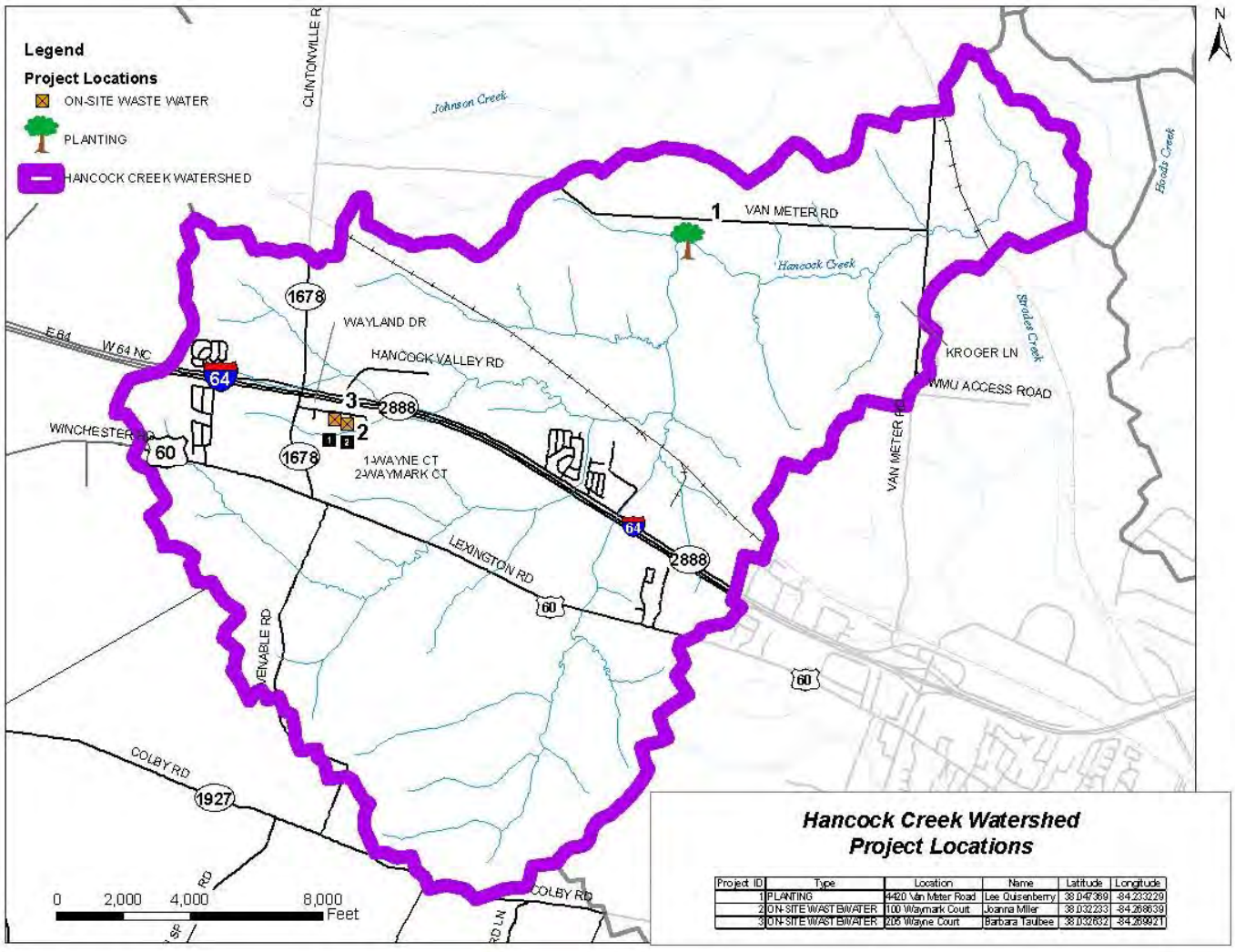

Figure 6. Onsite Wastewater and Tree Planting Project Locations.

Education

A number of water quality education classes were taught to elementary students through 4-H environmental camps during the course of the project. The students were quizzed by their teachers on the material presented to them during environmental camp. SCC staff used the Enviroscape to educate students about nonpoint source pollution. In addition to using the Enviroscape, SCC staff also used lessons from the Project WET Curriculum & Activity Guide (PWCAG). The lessons taught from PWCAG include Water Match (page 50), Imagine (page 157), The Incredible Journey (page 161), and Macroinvertebrate Mayhem (page 322). The following areas of the Core Content for Science Assessment Version 4.1 were covered:

1. SC-04-1.1.1- States of Matter
2. SC-04—4.6.1-Ecosystems & The Food Chain
3. SC-04—4.6.2-Sun's Light & Heat Are Necessary to Sustain Life on Earth
4. SC-04-4.7.1-Interdependence
5. SC-04-4.7.2-Human Interactions in the Environment Where They Live.

The project also helped Boy & Girl Scouts with various badge requirements that related to the natural environment.

Stream Event

The SCC sponsored a day of celebration showcasing TBSRP. Approximately 80 boy and/or girl scouts and their chaperones visited the stream on October 27, 2013. The SCC staff has received a number of positive comments about the event, including that it should be an annual scouting event. The stream event was relatively easy to put together because the duties were shared by four presenters. The format for a stream event is now developed, which would make an annual event quite easy. The City of Winchester purchased liability insurance for the day to cover costs of accidents and injuries.

Figure 7. Photo of project engineer explaining the riparian restoration to scouts.


Figure 8. Photo of instructor discussing bird flyways with scouts.

Low Impact Development (LID) Subdivision Regulations
This project paid for the drafts and final versions of the subdivision regulations that include an option for developers to build in a low impact way that reduces pervious pavement and encourages green infrastructure. At the time of this report, no development plans have been submitted for review for traditional or LID subdivisions. At least in the future, developers will have more choices for making neighborhoods that can be both aesthetically pleasing and more environmentally friendly. Please see Appendix G for the updated subdivision regulations.

Hancock Creek Watershed Plan
This project allowed for the HCWP to be updated to include:
1. The results of the MST monitoring,
2. A revised BMP Implementation Strategy, and
3. The load reductions expected as a result of the installation of BMPs. Please see Table 5.

The revised HCWP has been submitted to KDOW for comment and acceptance.

BMP	N Reduction	P Reduction	BOD Reduction	Sediment Reduction
	lb/year	lb/year	lb/year	t/year
Tree Plantings	1227.9	274.8	3253.8	184.2
Septic System Upgrades	93.3	36.5	380.8	0.0
Total	1321.1	311.3	3634.6	184.2

Table 5. STEP L Load Reductions.

Strodes Creek Conservancy Membership
The SCC continues to retain a number of its original members. The group continues to meet to discuss ideas to engage stakeholders in the watershed and discuss projects for improving water quality.

Stormwater Treatment System
The debris that will accumulate in the Stormsack will be removed by the COW Public Works Department's street sweeper truck. The unit has not been installed long enough at the writing of this report to determine the volume of debris that the unit will collect. However, manufacturer representatives recommend removing the accumulated debris biannually.

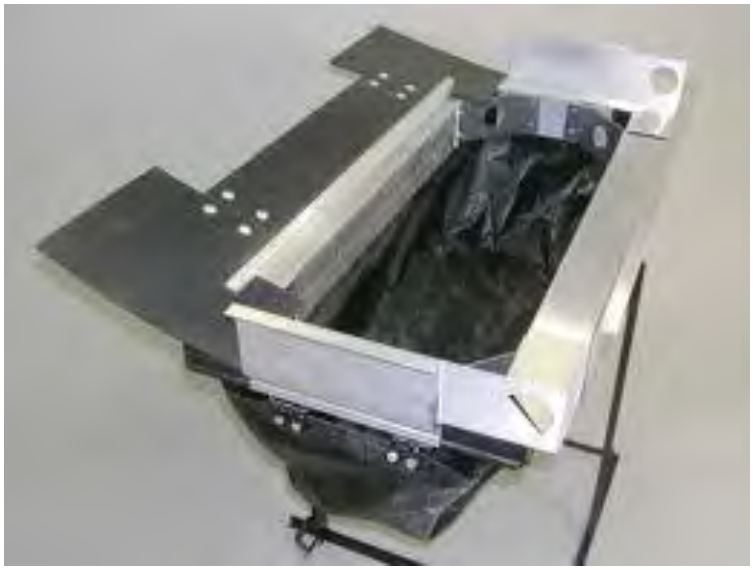

Figure 9. Photo of Fabco Stormsack

Conclusions

The COW was grateful to receive this grant so that they could continue the work started in the Strodes Creek watershed. This grant was exciting and useful because we were able to determine where our sources of pollutants originated. Previous work in the Strodes Creek watershed left a number of questions and speculation as to where funding dollars and administrative resources should be spent. Now, through the sampling data collected as a result of this grant, the SCC can continue to look for ways to get agricultural BMPs on the ground in the watershed. This grant also was able to install LID and green infrastructure language into the subdivision regulations, which is a source of great satisfaction. Making changes to subdivision regulations can often times be contentious and met with opposition. The LID and green infrastructure language included in the subdivision regulations allows for more flexibility for developers and have been proven to be cost effective. The grant's measures of success are discussed in more detail below.

Measures of Success
Goal 1: Refine the current Hancock Creek Watershed Plan to meet requirements for KDOW acceptance.

Objective 1: Collect additional data to fill identified data gaps and complete data analysis.

Measures of Success:

- Completion of the KDOW approved QAPP.

A QAPP for the collection of bacteria samples, discharge measurements (flow), and physical chemical water quality data in wadeable streams was approved by KDOW. The bacteria samples to be collected include Escherichia or E. coli, human-specific E. faecium and Bacteriodes, and cattle-specific Bacteroides. The physical chemical data collected include dissolved oxygen, pH, turbidity, conductivity, and temperature. The QAPP was approved by KDOW on August 8, 2011 and can be found in Appendix B.

- Completion of the monitoring in accordance with KDOW approved QAPP.

Monitoring was completed in the 2011 recreation season. The HC Monitoring & Microbial Source Tracking Report can be found in Appendix D. A deviation from the original QAPP was requested from KDOW to omit two of the samples, one wet weather and one dry weather. The summer and fall of 2011 were extremely dry with few rain events. The sampling team attempted their initial sample for the project for a dry event. However, conditions were too dry and several sites were ponded or had no flow. Since the sampling season was coming to an end, the sampling team collected a wet event sample on September 7, 2011 and followed up with a dry event sample on October 7, 2012. Because of this, the order in which the samples were to be taken was reversed and the project was able to conduct sampling and analyses for only one dry weather and one wet weather event.

Lessons Learned:
It is important to collect samples as early in the recreation season as possible in case weather conditions worsen as the summer wears on. Ensure that the samplers are readily available to sample throughout the recreation season. Before hiring the sampler or completing a sample schedule, check with sampler's vacation schedule and other work obligations.

- Completion of the data analysis for the Hancock Creek Watershed Plan.

The data analysis for the HCWP was completed and submitted to KDOW for approval. The completed Hancock Creek Monitoring & Microbial Source Tracking Final Report can be found in Appendix D.

Objective 2: Revise the BMP Implementation Strategy in the Hancock Creek Watershed Plan based on the completed data analysis.

Measure of Success:

- Completion of the revised BMP implementation strategy based on the current data analysis.

The sampling data collected during the project showed that cattle-specific Bacteroides were present in the majority of the samples. This result is not surprising, since pasture represents approximately 80 percent of the land use in the watershed. In addition, aerial photographs of pasture land in the area shows a wide range of relative vegetation densities, due to variable cattle stocking rates (i.e., animals grazing per acre) and pasture management practices.[7] The HCWP had already identified the need for pasture renovation/management and fencing and alternative watering systems.[7]

The sampling data collected during the project also showed the presence of E. coli, human-specific E. faecium, and Bacteriodes, all of which indicate the presence of human sources of bacteria. The HCWP had also already identified the need to locate and address leaking and failing septic systems and educate homeowners on septic maintenance.[7]

The BMP implementation strategy was not necessarily revised but prioritized. The agricultural BMP program was offered first. That program was not widely received by landowners. The onsite wastewater program was then offered to residents. The project helped install low pressure dosing systems at two homes in the Wayland Heights Subdivision.

Objective 3: Complete the revisions for the Hancock Creek Watershed Plan and submit for KDOW acceptance.
Measures of Success:

- Completion of the KDOW accepted Hancock Creek Watershed Plan.

The HCWP is an iterative document and will be revised and updated as activities in the watershed improve or impair Hancock Creek. The latest revision to the HCWP was completed in January 2013. The document was revised to include the results of the Center for Watershed Protection's Codes and Ordinances Worksheet, the updated, local subdivision regulations that now allow for low impact development, green infrastructure, and stormwater friendly growth, the Hancock Creek Monitoring & Microbial Source Tracking Final Report, and the estimated load reductions to the stream as a result of tree planting and onsite wastewater bmp implementation. Please see Appendices 2, 3, & 7.

Goal 2: Implement the identified activities of the KDOW accepted watershed plan for Hancock Creek.

Objective 1: Work with the SCC, local landowners and applicable agencies to implement the BMPs identified in the watershed plan as funding allows.

Measures of Success:

- Completion of BMP Implementation as funding allows throughout the Hancock Creek Watershed.

The HCWP identified four top priorities for BMP implementation for the community. The SCC, with the help of this project, was successful in working on two of the priorities. The priorities were:

1. Ordinance Assessment,
2. Pasture Renovation and Management,
3. Fencing and Alternative Watering Systems, and
4. Low Impact Development and Stormwater Education.

The SCC, Winchester-Clark County Planning Commission, and CDP Engineers, Inc. completed the Center for Watershed Protection's Codes and Ordinances Worksheet, which allowed the community to assess its ordinances. Based on the Center for Watershed Protection's Codes and Ordinances Worksheet, the Winchester-Clark County Planning Commission updated the local subdivision regulations to allow for low impact development, green infrastructure, and stormwater friendly growth. The project also paid for the drafts and final versions of the subdivision regulations that include an option for developers to build in a low impact way that reduces pervious pavement and encourages green infrastructure. The updated subdivision regulations can be viewed in Appendix G.

The SCC, with the help of this project, offered assistance to landowners for pasture renovation and management and fencing and alternative watering systems but did not have adequate interest to implement the BMPs.

Although not a top priority of the HCWP, maintaining onsite wastewater systems and planting riparian zones are listed as desired BMPs in the plan. As mentioned earlier, the SCC offered assistance with onsite wastewater (septic system) upgrades. The project successfully installed two low pressure onsite wastewater systems. To develop riparian corridors, thirty (30) balled and burlaped trees and approximately 300 sapling trees were planted along Hancock Creek.

Lesson Learned: Onsite wastewater installations are dependent on the weather and require dry conditions. Getting the systems installed in a timely manner can be difficult if the weather tends to be rainy. Contractors like to be paid for materials before the project begins.

Lessons Learned: Riparian plantings can be difficult to get established. Before planting trees anywhere, always call Kentucky's 811 or Before You Dig hotline to determine where underground utility lines are buried. Also, if there is a chance that saplings will come in contact with a mower, do not plant them. Even if mowing crews are alerted to presence of the newly planted saplings, there is a good chance that they will get mowed down. As for planting the preferable balled and burlapped trees, it is important that these trees are planted in March or November. The stress on the trees is obvious even when planted as late as April. Ensure that trees will be watered during dry periods for at least the first two years. Also, we learned that our contractor's equipment could not get as close to the stream as we would have liked in some situations.

The project administrators worked diligently to find funding for restoration projects. They secured $20,000 in grant monies from Kentucky American Water Company and Bluegrass PRIDE to construct six (6) naturally appearing and functioning wetlands totaling approximately 4 acres along Town Branch in Winchester, KY.

- Completion of education and outreach efforts throughout the watershed.

The SCC staff used a variety of outlets to educate the community about watersheds and water quality. For instance, the staff instructed approximately 300 students annually in watershed education using the Enviroscape and Project WET lesson plans. SCC newsletters were mailed to every household in the watershed. Please see Appendix H for an example. All of the large cattle producers in the watershed were personally contacted and offered best management practice (BMP) assistance. The project encouraged the funding and implementation of rotational grazing, stream exclusion fences, shade structures, and livestock alternative water supplies. The SCC has a webpage on the City of Winchester's website. The SCC's website address is http://www.winchesterky.com/index.aspx?NID=866. You can also access the website by going to the City of Winchester's website and hitting the link to the Strodes Creek Conservancy. Presentations on water quality were given to the Winchester Kiwanis Club, Winchester City Commission, Cub and Boy Scout Troops, and Girl Scout Troops.

Lesson Learned: A newsletter should be mailed frequently to keep constituents engaged.

Goal 3: Continue to build organizational strength to ensure that the SCC will exist beyond the timeline of the grant.

Objective 1: Effectively administer the project and continue to strengthen the SCC membership to maximize effective project management.

Measures of Success:

- Maintain current membership and add new members to the SCC.

The SCC has a number of individuals with a keen interest in water quality on its board. As individual's time becomes constrained with other commitments, it is increasingly important to retain those individuals and recruit other board members. Our current membership has been active for nearly nine years. We would like to see more homeowners and landowners in the watershed express interest in being on the board.

Works Cited

[1]http://www.nwf.org/Be-Out-There/Why-Be-Out-There/Benefits.aspx

[2]Study: Weinstein, N., Przybylski, A. K., & Ryan, R. M. (2009). "Can nature make us more caring? Effects of immersion in nature on intrinsic aspirations and generosity." Personality and Social Psychology Bulletin, 35, 1315-1329.

[3]KY Division of Water. "Water Quality Standards. Last modified April 29, 2010. http://www.water.ky.gov/sw/wqstandards.

[4]Strodes Creek Conservancy Sampling Data. Shanda Cecil. 2011.

[5]Morehead State University Sampling Data. Geoff Gearner. 2011.

[6]Texas Agricultural Extension Service. Onsite Wastewater Treatment Systems. Pub. No. L-52358-99. http://agpublications.tamu.edu/pubs/ewaste.

[7]Hancock Creek Watershed Team, Kentucky Waterways Allicance, and KY Division of Water. "Hancock Creek Watershed Plan". June 2010.

STEVEN L. BESHEAR
GOVERNOR

ENERGY AND ENVIRONMENT CABINET
DEPARTMENT FOR ENVIRONMENTAL PROTECTION
DIVISION OF WATER
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FRANKFORT, KENTUCKY 40601
www.kentucky.gov

LEONARD K. PETERS
SECRETARY

August 8, 2011

To: Shanda Cecil
Project Manager
Strodes Creek Conservancy
P.O. Box 40
Winchester, KY 40392

RE: Quality Assurance Project Plan (QAPP) for the project 'Hancock Creek Watershed Improvement Initiative'.

Dear Ms. Cecil,

The Division of Water has reviewed your revised Quality Assurance Project Plan for the above referenced project. This letter will serve as the final acceptance for the QAPP.

If you have any questions, please contact the Technical Advisor Brooke Shireman or myself, at 502-564-3410.

Regards,

E-Signed by Lisa Hicks
VERIFY authenticity with ApproveIt

Lisa A. Hicks
Division of Water
Quality Assurance Officer

LAH:lah

C: Jim Roe
Brooke Shireman

Strodes Creek Conservancy

Post Office Box 40 Winchester, KY 40392 Phone & Fax (859) 745-4042

July 12, 2011

Ms. Lisa Hicks
KY Division of Water
200 Fair Oaks Lane
Frankfort, KY 40601

Dear Lisa:

Please find enclosed our revised quality assurance project plan. If you have any questions, please give me a call.

Thank you,

Shanda

Shanda P. Cecil

Quality Assurance Project Plan

Hancock Creek: Watershed Improvement Initiative

C9994861-07

Prepared By: Strodes Creek Conservancy
P.O. BOX 40
Winchester, KY 40392-0040
(859) 745-4042
scecil@winchesterky.com

Prepared For: Kentucky Division of Water (KDOW)
200 Fair Oaks Lane
Frankfort, KY 40601
(502) 564-3410
lisa.hicks@ky.gov

Date: May 17, 2011

Revision Date: June 28, 2011

Revision No.: 1

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SECTION A – PROJECT MANAGEMENT

A1. Title and Approval Sheet

Quality Assurance Project Plan
For Hancock Creek Watershed Improvement Initiative

Signatures:

Geoff Gearner/ Shanda Cecil — 6 July 2011 / 7-12-11
QAPP Authors — Date

Shanda Cecil/ Project Manager/Supervisor — 7-12-11
Date

Project Quality Assurance Officer — 6 July 2011
Geoff Gearner — Date

Nonpoint Source Section Supervisor — 8/9/2011
Jim Roe — Data

Lisa A. Hicks/Quality Assurance Officer, KDOW — 08/08/11
Date

Larry Taylor/Quality Assurance Manager, Kentucky — 08/15/11
Department for Environmental Protection — Date

SECTION A – PROJECT MANAGEMENT

A1. Title and Approval Sheet

Quality Assurance Project Plan
For Hancock Creek Watershed Improvement Initiative

Signatures:

Geoff Gearner/ Shanda Cecil QAPP Authors	Date
Shanda Cecil/ Project Manager/Supervisor	Date
Project Quality Assurance Officer Geoff Gearner	Date
Nonpoint Source Section Supervisor Jim Roe	Data
Lisa A. Hicks/Quality Assurance Officer, KDOW	Date
Larry Taylor/Quality Assurance Manager, Kentucky Department for Environmental Protection	Date

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Revision History

This page documents the revisions over time to the QAPP. The most recent iteration should be listed in the first space, with consecutive versions following. Signatures may be required for revised documents.

Date of Revision	Page(s)/Section(s) Revised	Revision Explanation
June 28, 2011	A1, A6, A7, A9, B4, B7, and B9	Numerous recommendations made during initial review.

A2. Table Of Contents

List Of Appendices

List of Tables

List of Figures

A3. Distribution List

Name: Geoff Gearner
Title: Professor of Biology
Organization: Morehead State University
Contact Information:
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Dept. of Biological & Environmental Sciences
Morehead State University
Morehead, KY 40351
606.783.2803
g.gearner@moreheadstate.edu
Responsible for Sample Collection, DNA Isolations, DNA Analysis, and Polymerase Chain Reactions

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Title: Project Coordinator
Organization: Strodes Creek Conservancy
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scecil@winchesterky.com
Responsible for Physical Chemical Water Quality Data and Stream Discharge Data

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Contact Information:
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(859) 276-3506
lisa.martin@microbac.com
Responsible for E-Coli Data

Name: Marilyn Rowe
Title: City Clerk
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mrowe@winchesterky.com
Responsible for Document Management

A4. Project / Task Organization

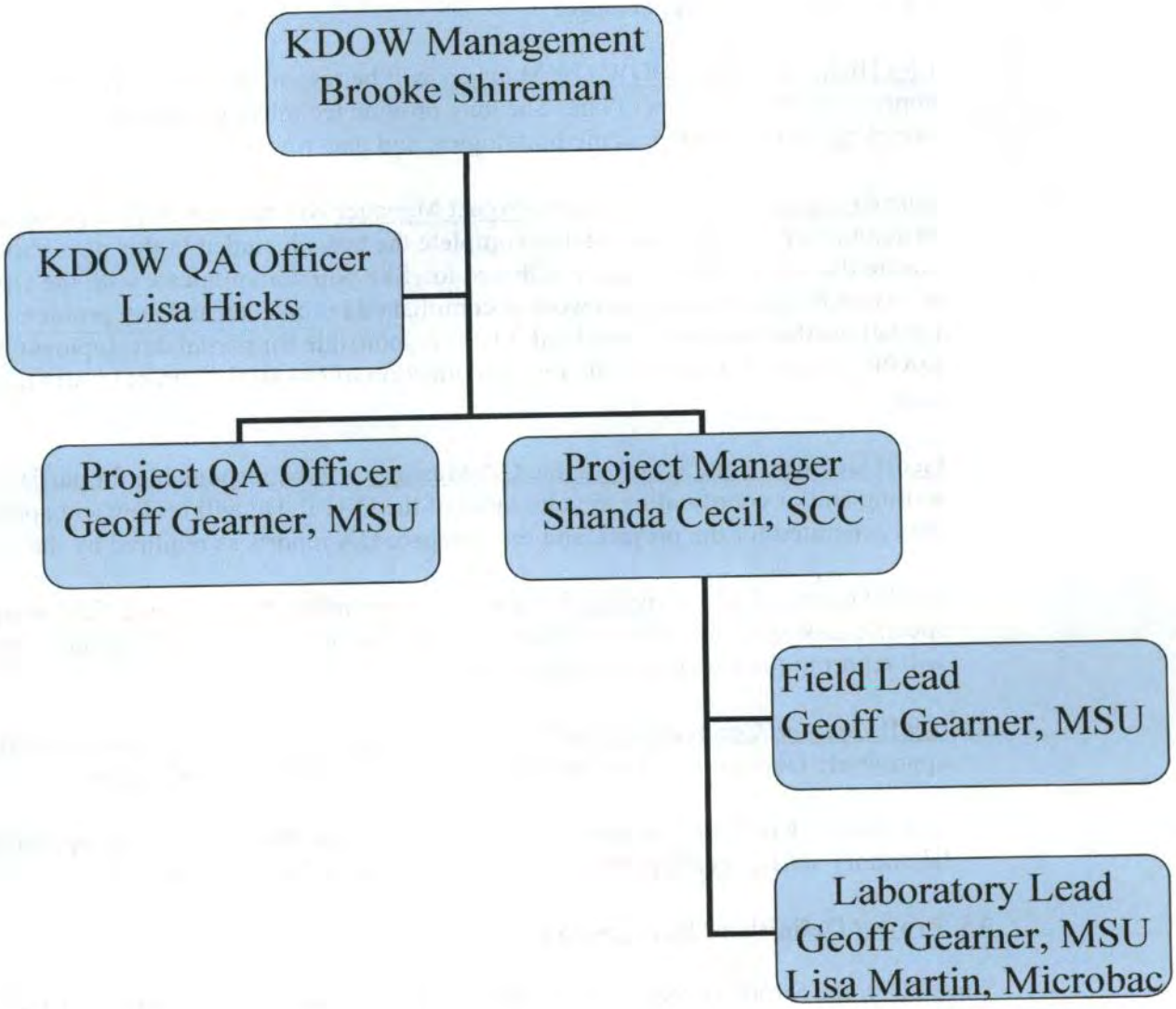


Figure 1. Project Organizational Chart

Brooke Shireman, KDOW Project Manager will be the responsible official for this project overseeing overall project operations and budget, as well as tasking contractors with work required to complete this project. She will communicate project needs to the contractor's project manager.

Lisa Hicks, Kentucky DOW QA Manager will be responsible for reviewing and approving the QA Project Plan. She may provide technical input on proposed sampling design, analytical methodologies, and data review.

Shanda Cecil, Contractor/Grantee Project Manager will have overall responsibility for assigning appropriate personnel to complete the tasks included in this plan. She will ensure that the project budget is adhered to. She will communicate with the Division of Water Project Manager on work accomplished in this plan and any problems or deviations that need to be resolved. She is responsible for partial development of the QAPP. She is also responsible for the collection of physical chemical water quality data.

Geoff Gearner, Contractor/Grantee QA Manager will be responsible for partially writing and/or coordinating development of the QAPP. He will review and approve all data generated for the project, and may prepare QA reports as required by the project.

Geoff Gearner, Field Sampling Lead will be responsible for assigning field samplers specific tasks and objectives. He has overall responsibility for all field activities. He will report to the Contractor Project Manager.

Geoff Gearner, Contract Laboratory Lead or Contact will be responsible for assigning appropriate laboratory staff to perform the analyses specified in this plan.

Lisa Martin, Contract Laboratory Lead will be responsible for assigning appropriate laboratory staff to perform the Colilert analyses specified in this plan.

A5. Project Definition / Background

Since 2004, the Strodes Creek Conservancy has been working in the Hancock Creek watershed through sample collection and analysis, best management practice (bmp) implementation, and stakeholder education. The watershed is quite diverse with agricultural land, scattered onsite wastewater systems, four package treatment plants, interstate runoff, a golf course, and scattered commercial and industrial activity. The resulting sample data has had consistently high bacteria counts. Typically, the SCC has helped land and home owners with agricultural and onsite wastewater bmps and riparian tree planting. As the SCC has become more aware of the water quality monitoring technology available today and the necessity to be good stewards of the funding afforded them, they have determined it is necessary to engage in microbial source tracking to determine if the pollution sources are coming from humans or animals. With the knowledge garnered from the data, the SCC can better focus their energies on the source of the problem and expend funding dollars more judiciously.

The principle objective in this study is to determine if fecal contamination in eight selected sampling sites of the Hancock Creek Watershed is of human and/or cattle origin. This determination will allow for effective remediation within the project area. This will be achieved by utilizing polymerase chain reaction (PCR) to amplify DNA sequences that are unique to host-specific bacterial strains to a detectable level. Specific PCR primers to human-specific E. faecium and Bacteriodes, and to cattle-specific Bacteroides will be utilized. In the end, the sources of impairments will be prioritized and remediation measures will be recommended in a final report. In general, the investigation will last 10 calendar months from start to report preparation. In addition, the SCC hopes to determine the density of bacteria found at each of the sampling sites and prioritize bmp practices based on those findings.

A6. Project/Task Description

The project area includes selected sites within the Hancock Creek watershed (HUC #0500102-030) in Clark County, Kentucky. Hancock Creek drains 12.9 square miles of northwestern Clark County.

The field activities to be conducted will include the collection of bacteria samples, discharge measurements (flow), and physical chemical water quality data in wadeable streams. The bacteria samples to be collected include Escherichia or E. coli, human-specific E. faecium and Bacteriodes, and cattle-specific Bacteroides. The physical chemical data collected include dissolved oxygen, pH, turbidity, conductivity, and temperature. The physical chemical data will be collected using a YSI multiparameters probe. The flow will be measured with a SonTek/YSI FlowTracker®.

EPA-approved, sterile sample containers will be distributed to samplers prior to sampling along with a pre-printed chain of custody forms, sampling instructions, and sample delivery logistics information.

To collect samples for bacteria in wadeable streams, samplers will wade to the middle of the stream and dip the sterile sample container to a depth of four inches with the open end of the container facing upstream. If the stream exhibits a low flow, the sampler will push the mouth of the container upstream at this depth until the container is nearly full. The opened mouth of the container will at all times be upstream of the sample collector, sampling apparatus, and any disturbed sediments.

The samples will be immediately chilled in an ice chest at a temperature of 1° to 4°C for transport back to the Microbiology Lab and the Microbac Laboratory. All samples will be processed for the assessment of bacteria density and DNA extraction within six hours of collection.

The results of this project will be documented in annual reports and a final report to the KDOW. All sample forms, field data forms, field notebooks, lab forms, log forms, quality assurance reports and lab notebooks will be kept on file in the Microbiology Laboratory (Lappin Hall, Room 344) at Morehead State University (Microbiology Lab). Dr. Geoff Gearner, will maintain all reports submitted to Shanda Cecil and the KDOW. The results of

this project may be used for future research, publications and presentations. Results will be tabulated in Microsoft Excel spreadsheets (or other appropriate software), stored on two secured computers, and backed-up on appropriate electronic storage media. All calculations and statistical data analysis will be stored as described above and submitted in the final report. All documents for this project will be kept for a minimum of 5 years.

Table 1. Hancock Creek Project Schedule

Event	Project Schedule
1. Develop QAPP (with review & revision)	April 2011 til June 2011
2. Conduct Monitoring	July 2011 til October 2011
3. Conduct DNA Isolations	July 2011 til October 2011
4. Analysis of DNA and polymerase chain reactions	September 2011 til December 2011
5. Conduct Results Analysis	January 2012
6. Conduct Briefing Meetings	February 2012
7. Submit Final Report	April 2012

Schedule of Sampling Events

Eight sites in the Hancock Creek Watershed (Table 1) will be sampled between July and October 2011. The sites have been thoughtfully selected to include sampling locations in the upper reaches of the watershed to a location just before Hancock Creek flows into Strodes Creek. Since the sampling is primarily being conducted to help the SCC determine the sources of bacteria in the watershed, the sampling locations can be found below major residential subdivisions, agricultural areas, industrial activity, and a golf course. By sampling below the residential subdivisions and the agricultural areas, the SCC will get a better idea as to the origin of the bacteria. Depending on the upstream land use, individual tributaries will be sampled as well as below the confluence of a couple of tributaries. The sampling will occur on waters that are not currently listed as impaired on the 303(3) List of Impaired Waters. Four sampling events will occur: one following each of two dry weather events (a period of seven days with a total rain accumulation of less than 0.1 inch), and two immediately following a wet weather event that proceeds a dry weather event.

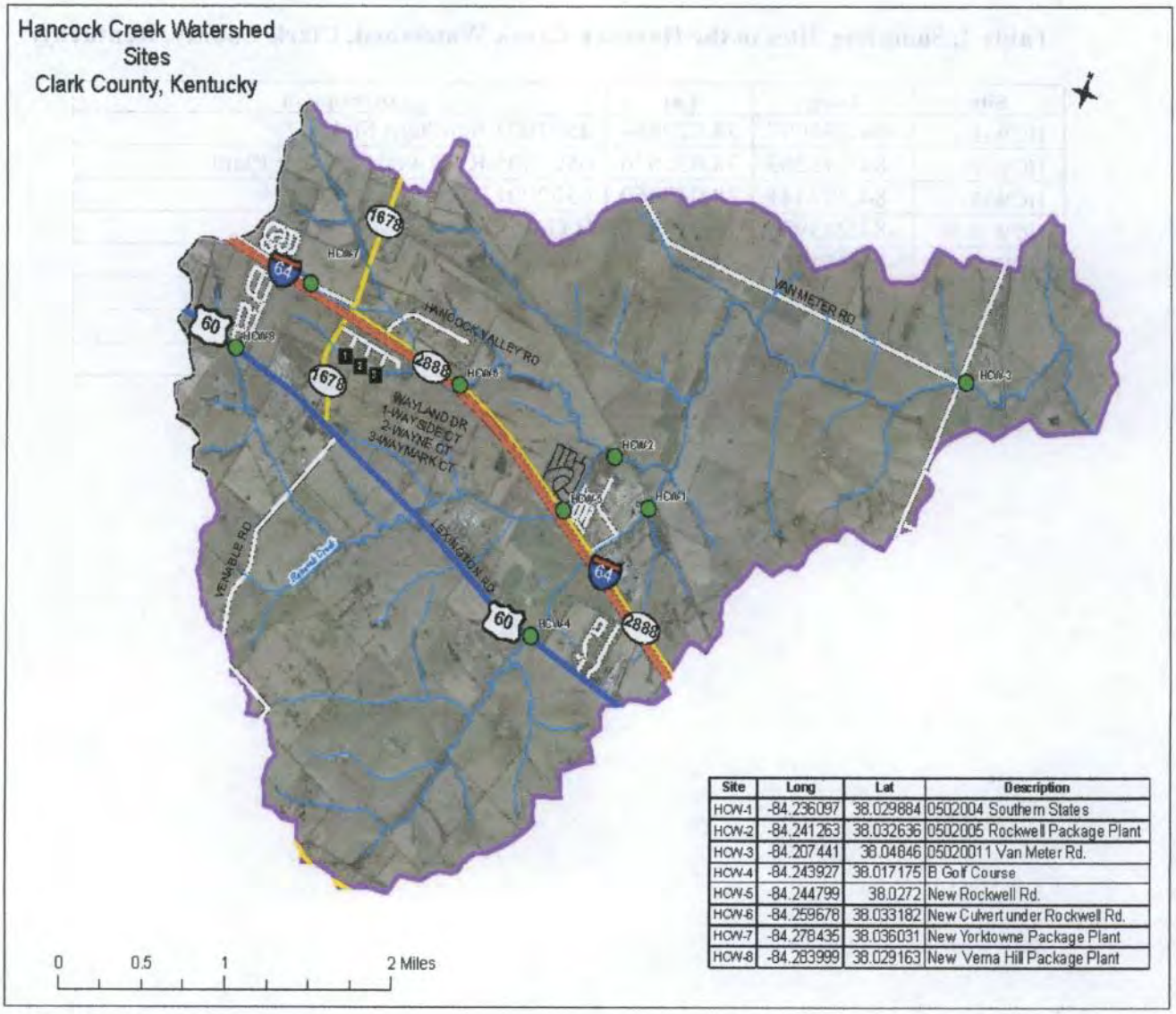
As mentioned earlier, in order to determine if pollution sources are of human or animal origin, new data will need to be obtained. The data necessary to address the pollution problem(s) within the Hancock Creek watershed include the utilization of polymerase chain reaction (PCR) to amplify DNA sequences that are unique to host-specific bacterial strains to a detectable level. Specific PCR primers to human-specific *E. faecium* and *Bacteriodes*, and to cattle-specific *Bacteroides*. In addition, the SCC hopes to determine the density of bacteria found at each of the sampling sites and prioritize bmp practices based on those findings. The bacteria densities found at each of the sample locations will also be helpful in correcting the pollution problem. Bacterial densities (E. coli) will be measured as per the Colilert method. For microbial source tracking, the samplers are interested in molecular markers from other feces-associated bacteria; however, that approach is qualitative, and not quantitative. Both the E. coli data and the MST data will provide information on the degree of fecal contamination present at a given sampling site, as well as the host source of that contamination. Together, those data will help inform the appropriate BMP to be implemented.

To maintain consistency with historical sampling data, E.coli and physical chemical water quality data will also be collected at each of the sites.

Table 2. Sampling Sites in the Hancock Creek Watershed, Clark County, Kentucky.

Site	Long	Lat	Description
HCW-1	-84.236097	38.029884	0502004 Southern States
HCW-2	-84.241263	38.032636	0502005 Rockwell Package Plant
HCW-3	-84.207441	38.048460	05020011 Van Meter Rd.
HCW-4	-84.243927	38.017175	B Golf Course
HCW-5	-84.244799	38.027200	New Rockwell Rd.
HCW-6	-84.259678	38.033182	New Culvert under Rockwell Rd.
HCW-7	-84.278435	38.036031	New Yorktowne Package Plant
HCW-8	-84.283999	38.029163	New Verna Hill Package Plant

Figure 2. Hancock Creek Sampling Sites



Site	Long	Lat	Description
HCW-1	-84.236097	38.029884	0502004 Southern States
HCW-2	-84.241263	38.032636	0502005 Rockwell Package Plant
HCW-3	-84.207441	38.04846	05020011 Van Meter Rd.
HCW-4	-84.243927	38.017175	B Golf Course
HCW-5	-84.244799	38.0272	New Rockwell Rd.
HCW-6	-84.259678	38.033182	New Culvert under Rockwell Rd.
HCW-7	-84.278435	38.036031	New Yorktowne Package Plant
HCW-8	-84.283999	38.029163	New Verna Hill Package Plant

A7. Data Quality Objectives (DQOs) and Criteria for Measurement Data

The overall project data quality objective (DQO) is to identify and quantify the sources of pathogen pollution to facilitate effective remediation. Reaching this objective requires that data generated and used for modeling must be of sufficient quantity and quality to support the identification of host sources in the area, the geographical location of host sources, and the quantification of the relative contributions of host sources.

For this project, measurements of *E. coli* counts and microbial source tracking data generated by polymerase chain reaction of host-specific bacterial/molecular markers will be collected and analyzed to assess the quality of streams both in their current state and after implementation of BMPs. DQIs are qualitative or quantitative descriptors of data quality. The

quality of field and analytical data is most often assessed in the following terms: precision, bias, accuracy, representativeness, comparability, completeness, and sensitivity. Table 4 lists the precision, accuracy and detection limits associated the laboratory instrumentation, equipment and culture media associated with *E. coli* density to be measured, and the detection of host-specific bacterial molecular markers utilized during this project.

Precision is the measure of agreement among repeated measurements of the same property under identical or substantially similar conditions; calculated as either the range or as the standard deviation. Precision uncertainties will be measured through the collection of field duplicate samples on 10 percent of the E-coli samples. The laboratory additionally performs duplicate sample analysis with each analysis batch. Precision in PCR and qPCR methods is ensured by testing multiple methodologies and through the use of triplicate analysis of DNA standards.

Bias is the systematic or persistent distortion of a measurement process that causes errors in one direction. Table 4, page 25, lists the biases of each method, if present, and the cause of these biases. For most methods, bias is incorporated into the uncertainty associated with the accuracy. For presence / absence PCR methods, false negatives can occur due to the small sample size and time period represented, but there is little to no generation of false positives.

Accuracy is a measure of the overall agreement of a measurement to a known value; it includes a combination of random error (precision) and systematic error (bias) components of both sampling and analytical operations. Accuracy will be determined through the use of quantitative samples of known value.

To complement this data, physical chemical water quality parameters will be tested. If an environmental monitoring system instrument is effectively calibrated, the measurements are reliable.

A8. Special Training Requirements / Certification

The microbial source tracking field sampling and laboratory analysis will be conducted by MSU scientist, trained MSU students, and trained volunteers. All volunteers and students will attend mandatory training. Community volunteers will be taught simplified yet approved sampling techniques for bacteria. The project manager (Dr. Geoff Gearner) will train MSU students. Sample analysis will be conducted by Dr. Gearner and his students working in MSU's Microbiology Laboratory. Student training includes safety in the Biosafety Level 2 Laboratory, basic aseptic techniques and microbiological practice, media preparation, field sample collection methods and transport, membrane filtration methods for bacterial assessment of water samples, polymerase chain reaction, and agarose gel electrophoresis, photodocumentation, and laboratory documentation and record keeping. All training records will be kept in three-ring binders in the Microbiology Laboratory. Dr. Gearner will verify that each sampler and laboratory participant has been trained. See the corresponding sub-sections of Group B for details related to sampling, lab methods and quality assurance.

The E-Coli samples will be collected by Shanda Cecil, a trained Licking River Watershed Watch volunteer.

A9. Documentation and Records

The results of this project will be documented in annual reports and a final report to the KDOW. In addition, a Quality Assurance Evaluation Report will be submitted to KDOW after the first sampling event and upon request by the KDOW. All sample forms, field data forms, field notebooks, lab forms, log forms, quality assurance reports and lab notebooks will be kept on file in the Microbiology Laboratory. In addition, raw data in the form of field sheets and/or calibration records will be available at the random request of KDOW and/or at the end of data collection. Dr. Geoff Gearner, will maintain all reports submitted to Shanda Cecil and the KDOW. The results of this project may be used for future research, publications and presentations. Results will be tabulated in Microsoft Excel spreadsheets (or other appropriate software), stored on two secured computers, and backed-up on appropriate electronic storage media. All calculations and statistical data analysis will be stored as described above and submitted in the final report. All documents for this project will be kept for a minimum of 5 years.

The most current QAPP, as well as any revisions or updates, will be distributed electronically to the appropriate project staff on the distribution list.

SECTION B. - DATA GENERATION AND ACQUISITION

B1. Sampling Process Design

Microorganisms

Bacteria are used as indicators of fecal contamination of waterways. Presently, E. coli, a bacterium associated with the feces of warm-blooded vertebrates (birds and mammals, including humans) is used an indicator organism. Other microbial indicators included *Enterococcus faecium* and anaerobic bacteria of the *Bacteroides-Prevotella* group. The principle objective in this part of the study is to determine if fecal contamination in eight selected sampling sites of the Hancock Creek Watershed is of human and/or cattle origin. This will be achieved by utilizing polymerase chain reaction (PCR) to amplify DNA sequences that are unique to host-specific bacterial strains to a detectable level. Specific PCR primers to human-specific *E. faecium* and *Bacteriodes*, and to cattle-specific *Bacteroides* will be utilized.

Physical Chemical Water Quality Parameters

Dissolved oxygen, temperature, conductivity, turbidity, and pH are used indicators of the overall health of a stream system. Unacceptable levels of any one of these parameters can have detrimental effects on the aquatic life in a stream or for one of a stream's many designated uses. The principle objective in this part of the study is to determine if the aforementioned water quality parameters are at levels in the eight selected sampling sites of the Hancock Creek Watershed.

Discharge Measurements

Stream flow, also called discharge, is a fundamental property of streams that affects everything from temperature of the water and concentration of various substances in the water to the distribution of habitats and organisms throughout the stream. Stream flow will be determined with rating curves developed for Hancock Creek and its tributaries subsequently used to calibrate flow. Discharge is determined using rating curves based upon a set of cross-sectional and discrete depth in-stream velocity measurements made with a FlowTracker® velocity meter over a range of discharge conditions. Using the FlowTracker® will allow the SCC to calculate instantaneous load as well as load concentrations.

B2. Sampling Methods

The field sampling and laboratory analysis for *E. faecium* and *Bacteriodes*, and to cattle-specific *Bacteroides* will be conducted by MSU scientist, trained MSU students, and trained volunteers. The field sampling for E-coli will be conducted by Shanda Cecil and analyzed by Microbac Laboratories, Inc. Physical chemical sampling of dissolved oxygen, pH, turbidity, and conductivity will be conducted by Shanda Cecil, the contractor project manager. Stream flow will be measured by Shanda Cecil, the contract manager, and a trained assistant. All sampling will follow the timelines and approaches summarized in Table 2 and in Section B1.

Eight sites in the Hancock Creek Watershed (Table 1) will be sampled between July and October 2011. Four sampling events will occur: one following each of two dry weather events (a period of seven days with a total rain accumulation of less than 0.1 inch), and two immediately following a wet weather event that proceeds a dry weather event.

Bacteria

EPA-approved, sterile sample containers will be distributed to samplers prior to sampling along with a pre-printed Chain of Custody forms, sampling instructions, and sample delivery logistics information.

To collect samples for bacteria in wadeable streams, samplers will wade to the middle of the stream and dip the sterile sample container to a depth of four inches with the open end of the container facing upstream. If the stream exhibits a low flow, the sampler will push the mouth of the container upstream at this depth until the container is nearly full. The opened mouth of the container will at all times be upstream of the sample collector, sampling apparatus, and any disturbed sediments.

The samples will be immediately chilled in an ice chest at a temperature of 1° to 4°C for transport back to the microbiology lab and Microbac Laboratory. All samples will be processed for the assessment of bacteria density and DNA extraction within six hours of collection.

Physical Chemical Water Quality Paramters

Dissolved oxygen, pH, turbidity, and conductivity will be measured using a YSI 6-Series, 6920 Sonde.

Stream Flow

Stream flow will be measured using a SonTec/YSI FlowTracker® handheld device and a wading rod.

B3. Sample Handling and Custody Requirements

Evidence of proper sample collection and handling will be thoroughly documented as all information pertinent to a field study and/or sampling will be recorded in field notebooks and/or chain of custody forms (Appendix C), and laboratory notebooks and/or lab data sheets. This documentation will follow these guidelines:

1. Field records will be completed at the time the samples are collected,
2. Names of sample collectors and witnesses who are present will be recorded,
3. All entries will be signed, including date and time, by the sample collector.

In the field, the sample collector will immediately perform the following tasks to ensure sample integrity:

1. Sample containers will be sealed and marked with stream name, station (sample site), date, time, sample number, and name of sample collector(s).
2. Bacteria samples will be stored on ice for transport to the Microbiology Laboratory.

The map geographic locations are named by their sample number.

B4. Analytical Methods Requirements

E. coli
Samples will be analyzed in the Microbac Laboratory using the Colilert method described in Appendix E for the detection and enumeration of E. coli. The bacterial density of the water sample will be reported as the number of positive E. coli colony forming units per 100 mL of sample (CFU/100 mL). Please see Appendix A for the standard operating procedure for Protocol for Detection and Enumeration of E. coli in Environmental Water Samples.

The laboratory will maintain and have available all quality assurance documentation as called for in *Standard Methods* (APHA, 1998).

Microbial Source Tracking
To determine if the source of fecal contamination is human and/or cattle, two polymerase chain reaction methods which amplify molecular markers unique to human and cattle fecal bacteria will be employed. Please see Appendix B for the standard operating procedure for Protocol for Polymerase Chain Reaction Amplification of Host-Specific Bacterial DNA Sequences.

Water samples will be collected and transported to the Microbiology Laboratory. Ten 100-mL samples will be collected at each site for the detection of *Bacterioides-Prevotella*-specific DNA sequences.

The first method will utilize PCR primers that amplify the 16S rRNA gene of cattle- and human-specific *Bacteriodes-Prevotella* bacteria (Table 2; Bernhard and Field, 2000a and 2000b). Water samples will be filtered through 0.2-µm pore size Supor-200filters. The filters will be placed in sterile 50-mL centrifuge tubes containing 5 mL of lysis buffer (20 m*M* EDTA, 400 m*M* NaCl, 750 m*M* sucrose, 50 m*M* Tris; pH 9.0) and stored at -80°C. The PowerWater DNA Extraction Kit will be used to extract DNA from the samples as per the manufacturer's directions (MO BIO Laboratories). All DNA samples will be assessed spectrophotometrically for concentration and purity, and stored at -20°C.

Table 3. PCR primers utilized for the detection of host-specific bacterial DNA sequences.

Gene	Bacterium	Primer	Sequence	Host Source
16S rRNA	*Bacteriodies-Prevotella*	HF183F Bac708R	5'-ATCATGATGTCACATGTCCG-3' 5'-CAATCGGAGTTCTTCGTG-3'	Human
16S rRNA	*Bacteriodies-Prevotella*	CF128F Bac708R	5'-CCAACYTTCCCGWTACTC-3' 5'-CAATCGGAGTTCTTCGTG-3'	Cattle
esp	*Enterococcus faecium*	espF espR	5'-TATGAAAGCAACAGCACAAGTT-3' 5'-ACGTCGAAAGTTCGATTTCC -3'	Human

Each PCR reaction will contain the following components:

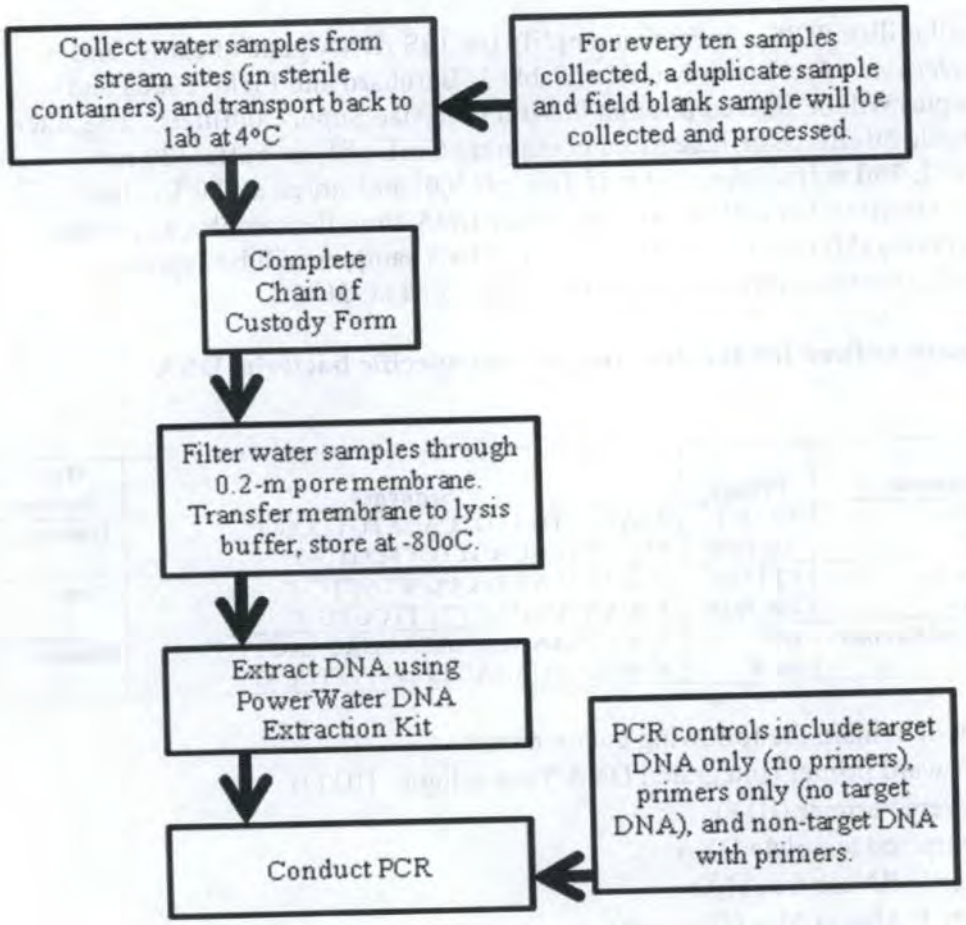
- 2.0 µL forward primer (Integrated DNA Technologies [IDT])
- 1.0 µL reverse primer (IDT)
- 1.0 µL extracted template DNA
- 6.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

The thermocycler conditions will be as follows:

Initial activation	2 min at 95°C
1. Denaturation	30 sec at 94°C
2. Annealing	60 sec at (59°C, HF183F) or (59°C, CF128F)
3. Extension	30 sec at 72°C
35 cycles of steps 1-3	
Final extension	7 min at 72°C

For each set of PCRs conducted, a primer-only control, target DNA template-only control, and non-target DNA control will be included. All PCR reaction products will be stored at -20°C until analysis. Figure 4 illustrates the workflow and quality assurance for PCR detection of *Bacteroides-Provetella* DNA sequences.

Figure 3. Flow Diagram of Sample Processing for Detection of Host-Specific Bacterioides-Prevotella DNA Sequences Quality Assurance in the Microbiology Laboratory.



The second method will utilize PCR primers that amplify the *esp* gene of human-specific *Enterococcus faecium* (Table 2; Scott, *et al.*, 2005).

Water samples will be collected and transported to the Microbiology Laboratory as previously described.

For each site-collected sample, 100-mL volumes will be filtered through a 0.45-µm pore size sterile membrane filter by vacuum aspiration. Membranes will then be transferred to modified mEI medium culture plates and incubated for 48 hr at 41°C according to EPA Method 1600 (USEPA, 2002a). Membranes containing enterococci colonies will be transferred to a sterile 50-mL centrifuge tube containing 20 mL of sterile tryptic soy broth, Vortexed, and incubated at 41°C for 3 hours to wash the bacteria off the membrane and partially enrich the culture. The QIAamp DNA Extraction Kit will be used to extract DNA from the samples as per the manufacturer's directions (Qiagen). All DNA samples will be assessed spectrophotometrically for concentration and purity, and stored at -20°C.

Each PCR reaction will contain the following components:

- 1.0 µL forward primer (0.3 µM; IDT)
- 1.0 µL reverse primer (0.3 µM; IDT)
- 5.0 µL extracted template DNA
- 5.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

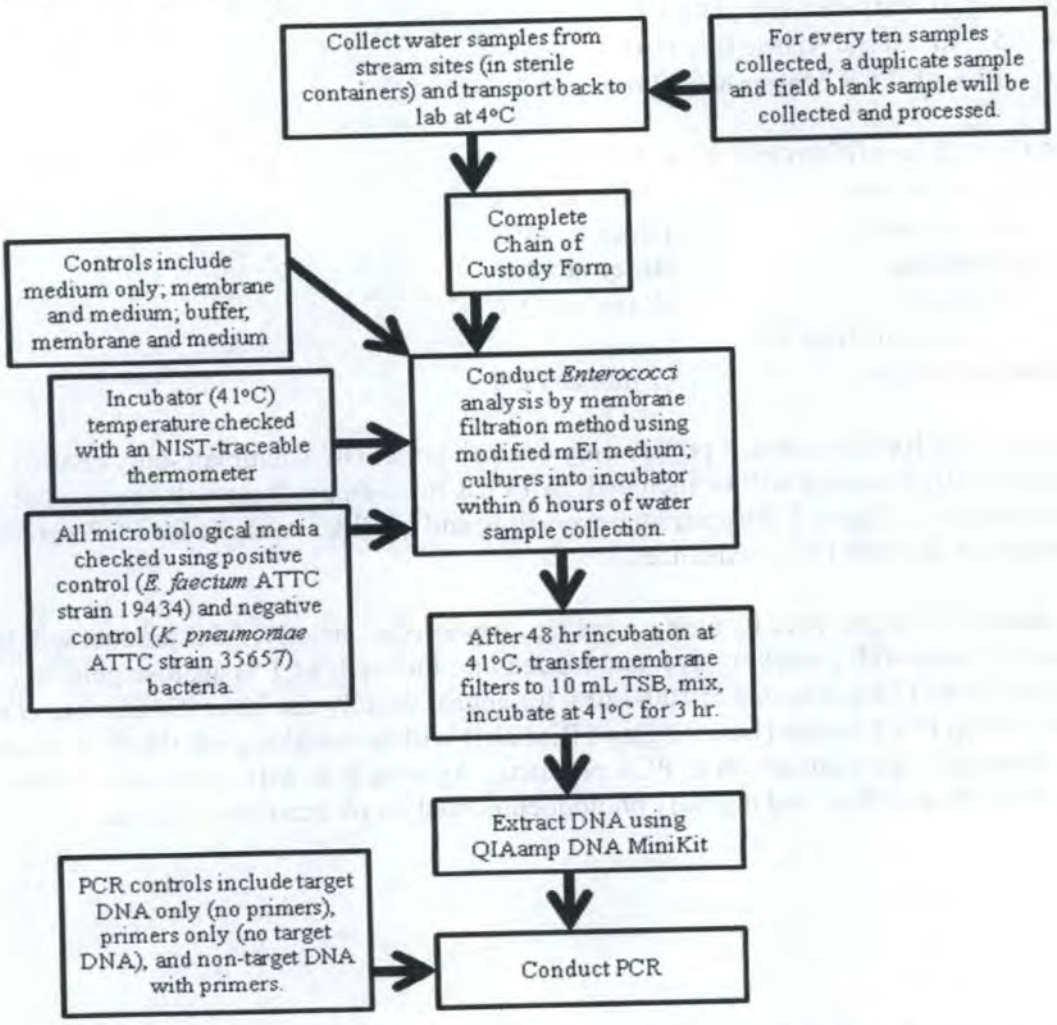
The thermocycler conditions will be as follows:

Initial activation	2 min at 95°C
1. Denaturation	15 sec at 94°C
2. Annealing:	60 sec at 58°C
3. Extension	60 sec at 72°C
35 cycles of steps 1-3	
Final extension	7 min at 72°C

For each set of PCRs conducted, a primer-only control, target DNA template-only control, and non-target DNA control will be included. All PCR reaction products will be stored at -20°C until analysis. Figure 5 illustrates the workflow and quality assurance for PCR detection of *Enterococcus faecium* DNA sequences.

PCR products will be analyzed by agarose gel electrophoresis. Briefly, PCR product will be mixed in a 1:1 ratio with a tracking dye, and loaded into the wells of 1 % agarose gels. Electrophoresis will be conducted at 100-120 v for approximately one hour (Sambrook, *et al.*, 1989). A 100-bp DNA ladder (New England BioLabs) will be run alongside the PCR product wells for base-pair size comparison to PCR products. Agarose gels will be stained with an ethidium bromide solution, and digitally photodocumented on uv transilluminators.

Figure 4. Flow diagram of sample processing for detection of host-specific Enterococcus faecium DNA sequences quality assurance in the Microbiology Laboratory.



Address issues of failed calibration checks or contamination in the analytical data. Identify the responsbible individuals for corrective actions, and the reporting official for the data and its limitations.

The Microbiology Laboratory will calibrate balances and pH meters according to the QAPP. Should a balance or pH meter fail to calibrate, the Microbiology Laboratory will use a different calibrated instrument. We will also make sure that incubators are maintained at the appropriate temperature by checking before and after incubating cultures with an NIST-traceable thermometer. If the incubator's temperature went above or below the uniformity boundary (see the previous table) during the incubation of bacterial cultures, then the Microbiology Laboratory would have to qualify the data in its report to you. If possible, the bacterial assessment will be repeated. Likewise, if any of the sample collection containers, member filters, culture dishes, culture media, or rinse buffer demonstrate microbial contamination (as assessed using previously mentioned controls), then the data could not be accepted and the Microbiology Laboratory would report that to you. If possible, the

assessment will be repeated. For the polymerase chain reaction method used in the microbial source tracking work, the data generated is acceptable as long as the experimental controls produce expected results. If the controls do not perform as expected, then the PCR experiment will be repeated.

Table 4. Methods, Ana ytes, and Data Quality Indicators for the Hancock Creek Watershed

Analyte	Methodolgy	Units	Precision	Bias	Accuracy	Comparability
Turbidity	YSI 6 Series, 6920 Sonde	NTU	10	See Accuracy	20	High Using Same Method
pH	YSI 6 Series, 6920 Sonde	S.U.	5	See Accuracy	5	High Using Same Method
Dissolved Oxygen	YSI 6 Series, 6920 Sonde	Mg/L	10	See Accuracy	20	High Using Same Method
Temperature	YSI 6 Series, 6920 Sonde	°F	5	See Accuracy	5	High Using Same Method
Conductivity	YSI 6 Series, 6920 Sonde	Umhos/cm	10	See Accuracy	10	High Using Same Method
E. Coli	Colilert	1/100 mLs		Acceptable	90%	High Using Same Method
Human & Cattle Specific Bacterioides-Prevotella	Polymerase Chain Reaction Amplification of Host-Specific Bacterial DNA Sequences.	Detection	N/A	False negatives can occur due to the small size and time period	Presence/ Absence	High with other presence / absense bacteroidetes tests, but may not detect inputs as old enterococcus tests
Human-specific Enterococcus faecium	Polymerase Chain Reaction Amplification of Host-Specific Bacterial DNA Sequences.	Detection	N/A	False negatives can occur due to the small size and time period	Presence/ Absence	High with presence/ absense enterrococcus tests

Definitions:
LOQ=Limit of Quantification

B5. Quality Control Requirements

E. coli

All micropipetters used will be calibrated annually to NIST standards.

All microbiological media and supplies, and all other reagents used in this project, will be purchased from the same suppliers to ensure consistency. All reagents will be labeled with a

receipt and opened date, lot numbers recorded, and stored according to the manufacturers' directions. All reagents will be used within the reported shelf life. The adequacy of microbiology media and biochemical reagents used for water testing and *E. coli* isolations will be determined by the responses of a positive control reference strain of *E. coli* (ATCC 25922) and negative control reference strain of *Klebsiella pneumoniae* (ATCC 35657) tested on the media for which typical responses are known.

A reference strain of *E. coli* (ATCC 25922) and of *E. faecium* (ATCC 19434), for which typical responses are known, will serve as a positive control for all selective and differential media and biochemical tests used in the isolation and identity confirmation of *E. coli*. A reference strain of *K. pneumoniae* (ATCC 35657), for which typical responses are known, will serve as the negative control for all selective and differential media and biochemical tests used in the isolation and confirmation of *E. coli*. One set of control bacterial strains will be inoculated onto each type of media on each day that water samples are tested for the presence of *E. coli*, and on each day that isolations and confirmation tests are performed.

Isolates obtained from watershed samples will be compared to positive (*E. coli* ATCC 25922) and negative (*K. pneumoniae* ATCC 35657) controls for which typical responses are known. Isolates that yield typical responses on all media and biochemical tests, compared to the *E. coli* reference strain, will become part of the microbiology lab's permanent culture collection for future analysis.

Table 5. Measures of Precision, Accuracy and their Detection Limits

Instrument	Precision	Accuracy	Detection Limits
Laboratory Instrumentation and Equipment			
pH meter	0.01 units	+/- 0.1 units	0 14 units
Balance	0.1 g	+/- 0.1g	0.001 g
37°C Incubator	Uniformity at 37°C +/- 0.5°C		
44.5°C Incubator	Uniformity at 44.5°C +/- 0.2°C		
P2 Micropipette	0.002 µL	+/- 0.013 µL	
P10 Micropipette	0.02 µL	+/- 0.075 µL	
P100 Micropipette	0.2 µL	+/- 0.4 µL	
P1000 Micropipette	2.0 µL	+/- 0.8 µL	
Pathogens			
Colilert		90%	Satisfactory
YSI Multiparameters Probe			
FlowTracker®	N/A	+/- 1% measured velocity*	N/A

*Resolution of 0.1 mm/second.

B6. Instrument/Equipment Testing, Inspecting and Maintenance Requirements

All equipment will be tested and inspected according to the manufacturers' recommendations. Each laboratory will be responsible for ensuring that the equipment is properly maintained. Each laboratory will record any testing, inspection and maintenance information for the equipment used. If it is determined that the equipment is malfunctioning, the issue(s) will be resolved before using the equipment in the Microbiology Laboratory or a different unit will be used that day.

B7. Instrument Calibration and Frequency

Balances will be calibrated using standard weights and built-in internal calibration procedures. Generally, modern balances with internal calibration require infrequent calibration, often only once per year if left on at all times.

The YSI 6 Series, 6920 Sonde will be calibrated according to specifications found in the YSI Environmental Operations Manual. The dissolved oxygen probe will be calibrated on the day of the sampling event. The pH probe will be calibrated at least every 14 days. The conductivity and turbidity probes will be calibrated monthly. See Appendix D.

No calibration of the FlowTracker®unit is required.

B8. Inspection / Acceptance Requirements for Supplies and Consumables

Bacteria

Only sample containers approved for use with the methods described above will be used during this project. The sampling event coordinator or a project manager will be responsible for distributing appropriate sample materials to supervising samplers or trained MSU students (or other trained volunteers).

For bacteria, sample containers will undergo a sterility check by the responsible project manager (Geoff Gearner). Nutrient broth medium will be introduced into the containers and incubated for 24 hours at 35°C. At least three containers will be checked per lot of 100. Membrane filters, media and other reagents will be tested prior to each sampling event for sterility.

B9. Data Acquisition Requirements for Non-direct Measurements

The SCC has sampled in the Hancock Creek watershed since May 2005. During that time, a significant amount of water quality data has been collected for sites HCW-1, HCW-2, HCW-3, HCW-4, and HCW-8. For comparison purposes, the historical water quality data will be included in the data set for this project.

GIS technology has been used for the creation of maps for this project. However, there are no plans to use GIS technology as a management tool for the rest of the project.

B10. Data Management

All chain of custody forms and biological assessment sheets will be kept in notebooks in the Microbiology Laboratory. Quality Assurance logs (i.e., incubator, refrigerators) will be kept in the Microbiology Laboratory.

All laboratory personnel will maintain a laboratory notebook. These notebooks will be the primary record of bacteriological assessments and DNA analyses, and will be kept in the Microbiology Laboratory. Data will be reviewed by the project manager (Gearner), and entered into an Excel™ spreadsheet.

All electronic documents will be kept on file in the Microbiology Laboratory. Back up files will be maintained in the office of the project manager (Gearner). All outputs produced in this project will be discussed and analyzed by the project manager and appropriate stakeholers in order to ensure that all of the data, results, and conclusions are accurate and appropriate. The results of this project will be shared with all stakeholders of the Hancock Creek Watershed Project. All electronic files will be reviewed and approved by all project managers before any document is considered final.

SECTION C – ASSESSMENT AND OVERSIGHT

C1. Assessments and Response Actions

The project manager (Gearner) and the appropriate stakeholers will jointly review data collection processes and sample analytic techniques. If necessary, corrective actions will be taken through supervisory controls or contract administration.

The types of assessments and responses to data collection activities are listed below. Each laboratory will maintain records related to field monitoring, laboratory testing, and data analysis.

- Field monitoring will occur on a continuous basis. Monitoring will include review of the project status and field records to ensure that project requirements will be met.
- Laboratory testing will occur on a monthly basis (according to SOP). Modification to QAPP, if necessary, will be requested in writing to the KDOW.
- Data analyses and continuous data will be reviewed by the project manager to ensure sufficient quality to develop a watershed based plan for the Hancock Creek Watershed.

C2. Reports to Management

The Quality Assurance Manager will notify the Contract Project Manager and KY DOW Project Manager biweekly on the status of the project, results of performance evaluations and systems assessments, results of data quality evaluations, and any significant quality assurance problems and recommended solutions.

Geoff Gearner, the Quality Assurance Manager, will prepare all reports. The Contract Project Manager will submit all reports to the KDOW.

SECTION D – DATA VALIDATION AND USABILITY

D1. Data Review, Validation and Verification

Laboratory data will be reviewed by the project manager (Gearner) within 30 days of its collection/processing. At this point the project manager will decide whether or not to accept the data

Laboratory data will be reviewed and verified for compliance with project requirements, and validated against the data-quality objectives listed in Section A7. Data that meet quality objectives defined by this project will be considered acceptable, and will be included in reports to the KDOW. Data that do not meet data quality objectives will be rejected and will not be included in statistical analysis, tables, and/or graphs.

Field staff, laboratory staff, and data entry staff are each responsible for verifying that all records and results they produce or handle are completely and correctly recorded, transcribed, and transmitted. The data will be spotted checked by the project manager (Gearner) and trained MSU students. Once the data have been checked, the spot checker(s) will initial the data sheets and/or laboratory notebook. Each project participant is also responsible for ensuring that all activities (sampling, measurements, and analyses) are performed with care

and diligence in order to produce the best quality sample analysis or data measurement possible.

D2. Validation and Verification Methods

All data reported via this project will be subject to checks for errors in transcription, calculation, and computer input. All field and laboratory data forms will be accurate and complete. Any changes to notes or data forms will be initialed and dated.

The staffs involved in the field, laboratory, and data management tasks are responsible for initial verification of the data that each task generates or handles. Verification of data from the Microbiology Laboratory will be accomplished using self-assessments and peer review by the project manager and/or trained MSU students. Outliers identified by the project manager will be examined for potential reasons for the unusual data distribution, or whether the data point is in error. The project manager will be responsible for resolving issues regarding outliers. If an issue cannot be corrected, the data associated with the issue will be rejected. All problems will be outlined in the appropriate notebook in each laboratory as well as any corrective actions taken. Correction actions may include, but not limited to, equipment maintenance or retraining of employees.

Data incorporated in the database will be reviewed and tested by the project manager.

D3. Reconciliation with User Requirements and Data Quality Objectives

Data will be continuously evaluated by the project manager (Gearner) MSU during the course of the project to ensure that it meets the quality objectives outlined in Section A7 of this document. If the data do not meet the goals specified in Section A7, they will not be included in graphs, relationships and equations reported to the KDOW.

Any suspected outliers in field or laboratory data will be re-sampled as soon as possible. Outliers will be determined by the project manager based on his collective knowledge of field sampling, laboratory analysis techniques, and local environmental knowledge. In addition, results that do not meet the quality assurance plan (i.e. not labeled properly or do not have a chain of custody form) will be re-sampled, if possible.

Following completion of the project all data will be stored either electronically or in paper format at Morehead State University for a minimum of 5 years.

SECTION E. - REFERENCES AND CITATIONS

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Institut Scientifiue d'Hygiene & Analyse. Validation Study of the Colilert®-18/Quantitray Method for the Enumeration of Escherichia Coliform & Coliform in Water for Human Consumption. 25 Avenue de la Republic. 91300 Massey.

APPENDIX A. E. COLI DETECTION/ENUMERATION PROTOCOL FOR DETECTION AND ENUMERATION OF E. COLI IN ENVIRONMENTAL WATER SAMPLES

Coliform Testing with Colilert ®

PREPARED BY:	Lisa Martin/Dee Cutrera/C.J. Lafollette
APPROVED BY:	Andrew Clifton
SUPERCEDES:	SOP Coliform and E. coli by Colilert 100301
REFERENCES:	*Commonwealth of Kentucky Manual for the Certification of Microbiology Laboratories Analyzing Drinking Water, Fifth Edition; SM 9223B*
APPLICATION:	Drinking and Surface Water

1. PRELIMINARY COMMENTS

1.1. This method is used to determine the presence of total coliforms and *Escherichia coli* (*E. coli*) in compliance drinking water samples, as mandated and regulated by the Environmental Protection Agency, the Kentucky Division of Drinking Water and the Commonwealth of Kentucky Laboratory Certification Authority. Also included in this SOP are all required and optional QA/QC procedures.

1.2. The laboratory must be certified for all analytical methods it uses for compliance purposes. In addition the laboratory must be certified enumeration of *E. coli* under the Long Term 2 Enhanced Surface Water Treatment Rule.

1.2.1. Registration with the EPA is also necessary to report results under the Long Term 2 Enhanced Surface Water Treatment Rule.

1.3. IDEXX training videos can be viewed @ http://www.idexx.com/view/xhtml/en_us/water/trainingvideos.jsf

2. PRESERVATION & HOLDING TIMES

2.1. Drinking water samples must be collected in a sterile, wide-mouth plastic bottle containing sodium thiosulfate. The bottle capacity must be at least 120 mL to allow at least one inch of headspace.

2.2. Samplers are encouraged, but not required, to hold drinking water samples at 0 - 10ºC during transport back to the laboratory. Source water samples as required by the Surface Water Treatment Rule are required to be held at 0 - 10ºC during transit. If samples are waiting to be analyzed the following day, they must stay in a refrigerator over night.

2.3. Samples are rejected for the following reasons:

2.3.1. Time between sample collection and receipt by laboratory being exceeded.
2.3.2. Presence of disinfectant in sample being noticed, e.g., odor
2.3.3. Evidence of freezing
2.3.4. Use of a container not approved by the laboratory for the purpose intended
2.3.5. Insufficient sample volume, e.g., <100mL
2.3.6. Presence of interfering contaminants, if noticed, e.g., hydrocarbons, cleansers, heavy metals, etc.
2.3.7. Sample temperature exceeding the maximum allowable

2.4. Samples must be analyzed within 30 hours for drinking water. Surface waters for LT2 must be analyzed with 8 hours of sample collection. All wastewater samples for E. coli must reach the lab within 6 hours of collection and set up within 2 hours of reaching the laboratory. Indiana pool samples must be analyzed with 24 hours.

2.5. All positive compliance sample trays must be stored in the microbiology fridge for at least one month from identification.

3. DEFINITIONS

3.1. Abbreviations and Acronyms

ASTM = American Society for Testing and Materials
ATCC = American Type Culture Collection
E. coli = *Escherichia coli*
D= dilution
HPC = Heterotrophic Plate Count
NH_3 = Ammonia
NIST = National Institute of Standards and Technology
PC = Plate Count
MUG = 4-methylumbelliferyl-β-D-glucuronide
MPN = Most Probable Number
QC = Quality Control
QA = Quality Assurance
RGW = Reagent Grade Water
SRGW = Sterile Reagent Grade Water
SOP = Standard Operating Procedure
TOC = Total Organic Carbon
TRC = Total Residual Chlorine
TSA = Tryptic Soy Agar
TSB = Tryptic Soy Broth
UV = Ultraviolet
CFU = Colony Forming Units
°C = degrees Celsius
mg = milligram
mg/L = milligram per liter
mL = milliliter
mm = millimeter
SU = Standard Units
PSI = pounds per square inch
$\mu W/cm^2$ = microwatts per square centimeter
µmho/cm = micromhos per centimeter

4. SAFETY CONSIDERATIONS

4.1. All samples or quality control tests suspected to contain any bacteria must be handled with gloves; contact with bacteria may cause serious infection or illness.
4.2. All samples or quality control tests known to contain any bacteria must be disposed of in a biohazards waste bag to be autoclaved.
4.3. All syringes must be disposed of in a Sharps Container to be autoclaved.
4.4. When removing items that are still hot from the autoclave, proper autoclave gloves must be worn.
4.5. Hands must be washed with anti-bacterial soap before and after performing any microbiology testing.

5. APPARATUS AND SUPPLIES

5.1. Equipment

Idexx Quanti-tray Sealer®
Dry Air Incubator, 35.0°C±0.5°C
Water Bath, 44.5°C±0.2°C
Water Bath, 55.0°C – 60.0°C
Balance
Conductivity Meter
pH Meter
Refrigerator,0-6°C
365 nm/6 watt Ultra-violet Light
Ultra-violet Light Meter
Idexx Quanti-trays®
Idexx Quanti-trays 2000®
Lysol® Brand II Disinfectant
47 or 57 mm Plastic, Sterile Petri Dishes
Aluminum Foil
Sterile Cotton Swabs
Alcohol burner
Weigh Boats
Antibacterial soap o
Biological Indicators

Dark Box
Colony Counter
NIST Thermometer
Pseudomonas aeruginosa
Thermometers
Autoclave
Bio-hazardous Waste Containers
Nickel Alloy/Platinum Inoculating Loops
Stir Bars
ASTM Class 1 Weights
Spatula

Escherichia coli
Citrobacter freundii
Sharps Containers
Blue Food Coloring
Total Residual Chlorine Packets
Colilert® Comparator
Autoclave Bags
Sterility Tape
Sterilization Indicator
Stopwatch
Sterile bottles 120 –mL, marked @ 100mL

5.2. Glassware

Erlenmeyer flasks
47 mm Glass Petri Dishes with Lids
Vials, Screw Top
Media bottles
Alcohol burner

1mL pipettes, Class B or better
Dilution Bottles, Screw Top
Graduated Cylinders, 100mL
10 mL Pipettes Class B or better

5.3. Facilities

This method must be performed in a dedicated room, separated from the rest of the laboratory, in order to prevent cross-contamination. Bench areas must be easily cleaned with disinfectant. Access to the micro laboratory is on an as needed basis. There must be sufficient space for processing samples; storage spaces for media, glassware, and portable equipment; floor space for stationary equipment; and areas for cleaning glassware and sterilizing materials

6. REAGENTS

6.1. Purchased Reagents

Chemical Name	Formula	Purity	Description
Amphyl®	NA	NA	o-Phenylphenol, o-Benzyl-p-chlorophenol
Bleach	NA	NA	Sodium Hypochlorite, Water, Sodium Hydroxide
Colilert®	NA	NA	Nutrient Indicator with MUG
Colilert®-18	NA	NA	Nutrient Indicator with MUG
Indole	NA	NA	0.5 mL 5% *p*-dimethylamino-benzaldehyde dissolved in a solution of 25% Hydrochloric Acid and 75% isobutyl alcohol
m Endo Agar LES	NA	NA	Yeast Extract, Casitone, Thiopeptone, Tryptose, Lactose, Potassium Phosphate Dibasic, Potassium Phosphate Monobasic, Sodium Chloride, Sodium Desoxycholate, Sodium Lauryl Sulfate, Sodium Sulfite, Basic Fuchsin, Agar
Methanol	CH_3OH	ACS Certified	NA
Ethanol	CH_3CH_2OH	NA	NA
Methane	CH_4	NA	NA
pH Buffer 4	NA	NA	NA
pH Buffer 7	NA	NA	NA
pH Buffer 10	NA	NA	NA
Plate Count Agar	NA	NA	Pancreatic Digest of Casein, Yeast Extract, Dextrose, Agar
Conductivity Calibration Standard	NA	NA	High Purity Water, Potassium Chloride, 1-propanol
Total Chlorine Reagent	NA	NA	Carboxylate Salt, Salt of N,N-Diethyl-p-Phenylenediamine, Potassium Iodide, Sodium Phosphate Dibasic
Tryptic Soy Broth	NA	NA	Pancreatic Digest of Casein, Enzymatic Digest of Soybean Meal, Dextrose, Sodium Chloride, Dipotassium Phosphate
Tryptic Soy Agar	NA	NA	Pancreatic Digest of Casein, . Enzymatic Digest of Soybean Meal, Sodium Chloride, Agar

6.2. Prepared Reagents

Name	Reagents Used	Amount Used	Procedure
50% Bleach	• Bleach • DI Water	• 100mL of Bleach • 100mL of DI Water	Add 100 mL of Bleach into 100 mL of DI Water.
2% Amphyl®	• Amphyl® • DI Water	• 4mL of Amphyl • 200mL of DI Water	Add 4 mL of Amphyl® into 200mL of DI Water.

7. STANDARDS

7.1. Escherichia coli ATCC 25922
7.2. Pseudomonas aeruginosa ATCC 10145 or 27853
7.3. Citrobacter freundii ATCC 8090

8. CLEANING CONSIDERATIONS

8.1 Any glassware that has been exposed to biohazard materials must be autoclaved for 30 minutes at 121°C / 15 PSI before being washed thoroughly SOP Laboratory Cleaning Practices.

9. SAMPLE PREPARATION

9.1. Samples must be filled to the 100-mL fill-line plus 2.5mL.
9.1.1. If sample is less than 100-mL, the client must recollect sample.
9.1.2. If sample is greater than 102.5mL, the analyst must aseptically pipette the excess sample into a separate, sterile sample bottle to be disposed. Opened packs of disposable sterile pipettes must be resealed between use periods.

10. PROCEDURE

10.1. Total Coliform Analysis

10.1.1. If reporting a MPN value, turn on Quanti-tray® Sealer and allow to warm-up. Sealer will be ready for use when green light is on.
10.1.2. Run one method blank of sterile reagent grade water and Colilert reagent with each run of samples.
10.1.2.1. To prepare the sterile reagent water, transfer DI water into an autoclavable container.
10.1.2.2. Loosely place a lid on container so pressure does not build up in container during autoclave cycle.
10.1.2.3. Autoclave for 15 minutes at 121°C / 15 PSI. If autoclaving more than 1L volumes, autoclave for 20 minutes.
10.1.2.4. At the end of cycle and when water has cooled, decant 50 mLs of sterile water into two sterile containers that are at least 100 mLs in size. Add 50 mLs of sterile double strength tryptic soy broth to both containers.
10.1.2.5. Incubate one bottle in air incubator for 48 hours at 35°C. Any turbidity in the bottle indicates bacterial contamination. If observed, batch should be discarded.
10.1.3. Label all Quanti®-trays or bottles with a corresponding sample identification name. Labeling should include client name, and location code. For LT2 samples label Quanti®-trays 2000 with a corresponding sample identification name.
10.1.4. Pour one packet of Colilert® into each sample bottle.
10.1.4.1.1. If Colilert 18 is used for P/A, add media to sample and then incubate for 7-10 minutes @ 44.5 °C. Be sure that the bottle is submersed at least ½ the depth of the container. (Trays are not prewarmed) Document that the incubation was performed in the appropriate log.
10.1.4.1.2. Sample temperature must be 35-37 °C when they are removed from the water bath. If not at the correct temp, place in water bath again while monitoring that the sample temp does not exceed 37°C.
10.1.5. Shake each sample bottle 30 times or until powder is completely dissolved.
10.1.5.1. When reporting presence/absence only, skip sections 10.1.6 and 10.1.7 and proceed to 10.1.8.

10.1.6. Slowly pour sample into corresponding Quanti®-tray. For LT2 samples, pour into corresponding Quanti®-tray 2000.
10.1.7. Place Quanti®-tray or Quanti®-tray 2000 containing sample into tray carrier and place into Quanti®-tray Sealer and seal tray shut. Repeat for each sample.
10.1.8. Incubate all samples in a 35.0 ±0.5°C incubator for 24-28 or 18-22 hours depending on media type.
10.1.9. Remove the samples from the incubator and interpret the results according to the procedures in Section 12.
10.1.10. If the sample is found to be total coliform positive, place sample under the ultra-violet light to detect the presence of *E. coli*. If any of the wells fluoresce, confirm the presence of *E. coli* according to section 12.3
10.1.11. All positive compliance sample trays or bottles, along with all confirmation tests, must be stored in the microbiology fridge for no less than one month.

10.2. Confirmation of *E. coli*

10.2.1. On back of the tray with *E. coli* positive sample, indicate the *E. coli* positive well(s) and swab with alcohol. Allow alcohol to air dry.
10.2.2. With a sterile syringe, extract ~ 0.5 mL of sample from an E. coli positive well or bottle.
10.2.3. Transfer the aliquot of *E. coli* positive sample into sterile test tube.
10.2.4. Add 0.5 mL of Indole to test tube with *E. coli* positive sample.
10.2.5. Interpret the results according to Section 12.
10.2.6. Indole confirmation is only required for Kentucky compliance drinking water.
10.2.7. Report positive samples per SOP Laboratory Response to Drinking Water Tests for Bacteria (Kentucky) or (Indiana)

11. QUALITY CONTROL

Document all temperatures, times, analyst initials, reagent IDs, lot numbers, QC checks, observations and results on the appropriate log book or log sheet.

Quality Controls: Purpose and Criteria

Quality Control	Description / Purpose	Frequency	Criteria
Bottle Sterility	This test ensures that all sample bottles are sterile, containing no bacteria contamination.	Per Lot Upon Receipt	no turbidity = no bacteria
Bottle Autofluorescence	This test ensures that all sample bottles have no autofluorescence that could cause a false *E. coli* positive.	Per Lot Upon Receipt	no fluorescence
Bottle Fill-line Check	This test ensures that all sample bottles have an accurate fill-line allowing the correct amount of sample to be collected for test performance.	Per Lot Upon Receipt	100 mL ± 2.5 mL
Colilert® and Colilert®-18 Media Sterility Check	This test ensures the sterility of all new lots of Colilert® and Colilert®-18 media.	Per Lot Upon Receipt and Monthly	no total coliform growth
Colilert® and Colilert®-18 Media Positive Control Check	This test ensures proper positive bacteria detectability for all new lots of Colilert® and Colilert®-18 media.	Per Lot Upon Receipt and Monthly and Monthly	MPN > 1.0 - < 200
Colilert® and Colilert®-18 Media Negative Control Check	This test ensures proper negative bacteria detectability for all new lots of Colilert® and Colilert®-18 media.	Per Lot Upon Receipt and Monthly	0 total coliform growth
Colilert® and Colilert®-18 Media Autofluorescence	This test ensures zero autofluorescence, which could cause a false *E. coli* positive, for all new lots of Colilert® and Colilert®-18 media.	Per Lot Upon Receipt and Monthly	no auto- fluorescence
Colilert® and Colilert®-18 Media pH check	To ensure proper pH range for all new lots of Colilert® and Colilert®-18 media.	Per Lot Upon Receipt and Monthly	pH range of 7.0 – 7.8
Quanti-tray® Sterility	All new lots of Quanti-trays® are checked for sterility.	Per Lot Upon Receipt	no bacterial contamination
Quanti-tray® Autofluorescence	All new lots of Quanti-trays® are checked for autofluorescence, which could cause a false *E. coli* positive	Per Lot Upon Receipt	no auto- fluorescence
Sealer Check	Once a month, Quanti-trays® are checked with 100 mL of blue dye water and ran through sealer to ensure there are no leaks in trays and sealer is sealing properly.	Monthly	no leakage
Reagent Grade Water Quality Assurance: pH	Once a month, the reagent grade water is tested for pH to make sure it is in the correct range.	Monthly	pH range 5.5 – 7.5
Reagent Grade Water Quality Assurance: Conductivity	Once a month, the reagent grade water is tested for conductivity to make sure it is in the correct range.	Monthly	Cond. range < 2.0 µmho/cm

Reagent Grade Water Quality Assurance: Chlorine	Once a month, the reagent grade water is tested for total residual chlorine to make sure it is in the correct range.	Monthly	Chlorine range < 0.02 mg/L
Reagent Grade Water Quality Assurance: Heterotrophic Plate Count	Once a month, the reagent grade water is tested for heterotrophic plate count to ensure it is less than 500 CFU.	Monthly	Bacteria range < 500 CFU
Incubator Check	Twice daily incubator temperature is recorded.	Twice Daily	34.5 >T<35.5
Microbiology Balance Check	Once a month, a set of specified weights is weighed to ensure balance is within the acceptance range.	Monthly	See Chart 8.2.15.2.
Conductivity Meter Quality Control Check	Once a month, the conductivity meter accuracy is checked with a low conductivity calibration standard.	Monthly	Cond. Range < 15 µs/cm
Autoclave Cleaning	Once a month, autoclave is cleaned.	Monthly	Must be free of debris
Autoclave Timer Check	Quarterly, the autoclave sterilization time is checked for accuracy.	Quarterly	Total cycle < 45 min
UV Reading	Once a month, the UV light is checked with an UV meter to ensure an accurate reading.	Quarterly	% Reduction > 70%
Lamp Cleaning	The UV light is cleaned monthly with ethanol.	Monthly	Must be free of debris
Sanitizer Swab Test	Once a month, a counter swab test involving Lysol®, Amphyll® and bleach is run to ensure the proper disinfecting performance of each.	Monthly	0 bacterial growth
Air Plate Monitoring	Once a quarter, two areas of the microbiology room are checked for air quality with plates of TSA.	Quarterly	0 bacterial growth
Bioindicator Check	Once a month, the sterilizing performance of the autoclave is checked with bioindicators.	Monthly	purple color
Stock Cultures	Cultures of *E. coli, Citrobacter freundii and Pseudomonas aeruginosa* are maintained for QC purposes. They are transferred each month to a new vial of TSB for 5 months in a row. After 5 months, the cultures are discarded and new cultures obtained. Quarterly purity checks are run on each bacterium.	Monthly and Quarterly	cultures must be properly cared for and pure
Temperature Distribution Studies	Annually, temperature distribution studies are performed on all water baths and incubators. This is to ensure even temperature distribution.	Annually	Temp. accuracy range: ±0.2°C
Thermometer Calibration	Once a year, each thermometer is calibrated against a NIST certified thermometer.	Annually	1°C± of NIST .
TOC, NH_3 and Silica on DI Water	Once a month, the TOC, NH_3 and silica of reagent grade water are tested to ensure the proper range.	Monthly	TOC <0.5mg/L NH_3 <0.1mg/L
Bacteria Suitability Test	Once a year, the reagent grade water is analyzed by an independent laboratory to ensure that it is under the correct bacteriological reporting limit.	Annually	Ratio of growth rate: 0.8-3.0
Heavy Metals on DI Water	Once a year, the reagent grade water is analyzed by an independent laboratory to ensure that it is within specification..	Annually	<0.05mg/L per contaminant <0.1mg/L total
UV Meter Calibration	Once a year, the UV meter is calibrated externally.	Annually	A Sca = 600µW/cm² B Sca = 3000µW/cm²
Autoclave Service	Once a year, preventative maintenance is performed on the autoclave.	Annually	119 - 124°C / 15 PSI
Inhibitory Residue Test	Once per product, an inhibitory residue test is performed to ensure that the microbiology soap is not inhibiting the growth of bacteria.	Annually	<15% colony count difference
Analyst Blind Samples	Twice a year, all certified microbiology analysts perform blind samples to demonstrate test performance capabilities.	Biannually	All analysts must pass blind samples.
Performance Evaluation	Once a year, the primary microbiology analyst performs HPC, drinking water and wastewater Performance Evaluation samples to ensure test performance capabilities.	Annually	Primary micro analyst must pass all PE samples.
0-5°C Refrigerator Temperature Checks	Once daily, the temperature of the refrigerator is checked to assure a temperature in the range of 0-5°C is maintained.	Daily	0-5°C

11.1. Bottle Sterility

11.1.1. Microbiology sample bottles sent to clients are purchased pre-sterilized; the sterility of each new lot of bottles is tested upon receipt.

11.1.2. Using aseptic technique, add 100mLs sterile tryptic soy broth to one bottle from the new lot.

11.1.3. Incubate the bottle at 35.0°C ±0.5°C for 48 hours.

11.1.4. Acceptable criterion is no observed turbidity.

11.1.5. If turbidity is observed, retest another bottle from the same lot. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer

11.2. Bottle Autofluorescence

11.2.1. The sterility of sample bottles is tested upon receipt with 366-nm ultraviolet light to ensure no fluoresce is observed.

11.2.2. Place a bottle on designated area (marked with tape) inside black box.

11.2.3. If the bottle fluoresces, notify the manufacturer and either discard or return the lot to the manufacturer.

11.3. Bottle Fill-line Check

11.3.1. Idexx bottles are pre-measured with a 100mL fill-line; upon receipt, the fill-line of one bottle from each new lot is verified.
11.3.2. Fill the new bottle to the fill-line with 100mL of RGW.
11.3.3. Pour the water from the bottle into a 100mL Class A graduated cylinder.
11.3.4. The acceptable volume criterion is 100.0 to 102.5mL.
11.3.5. If the measured volume is outside of the acceptable criterion, repeat the measurement for a second bottle from the lot. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer.

11.4. Colilert® and Colilert®-18 Positive Control Check

11.4.1. Proper positive detectability of Colilert® and Colilert®-18 is tested with two prepared positive control samples monthly or upon receipt of a new lot, which ever is sooner. One positive sample is prepared using *E. coli*, the other with *Citrobacter freundii*.
11.4.2. Using an inoculating loop, place one loop of bacteria into 100mL of SRGW in a dilution bottle.
11.4.3. Using a glass pipette, transfer 1mL of this solution into a dilution bottle containing 99mL of SRGW.
11.4.4. Transfer 1mL of this dilution into separate dilution bottle containing 99mL SRGW. This solution is a 10,000-fold dilution of the original solution. It shall be referred to as the positive control sample and is expected to yield a reasonable number of viable organisms (MPN < 2400).
11.4.5. Add one packet of Colilert® or Colilert®-18 to the positive control sample.
11.4.6. Shake the bottle 30 times and then pour into a Quanti-tray 2000®
11.4.7. Seal the tray using the Quanti-tray® Sealer.
11.4.8. Incubate the sealed Quanti-tray® for 18 hours for Colilert®-18 or 24 hours for Colilert®.
11.4.9. Remove the tray or bottle from the incubator and follow Section 13 to interpret the sample results. Acceptable results are within 1 to 2400 MPN.
11.4.10. If either of the two positive control samples are outside of the acceptable criteria, repeat the procedure for the failing control. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to manufacturer.

11.5. Colilert® and Colilert®-18 Negative Control

11.5.1. Proper negative detectability of Colilert® and Colilert®-18 is tested with negative control samples prepared with Pseudomonas aeruginosa monthly or upon receipt of a new lot, which ever is sooner.
11.5.2. Using an inoculating loop, add one loop of Pseudomonas aeruginosa into dilution bottle containing 100mL of SRGW.
11.5.3. Add one packet of Colilert® or Colilert®-18 to the bottle.
11.5.4. Shake the bottle 30 times and then pour into a Quanti-tray 2000®
11.5.5. Seal the tray using the Quanti-tray® Sealer.
11.5.6. Incubate the sealed Quanti-tray® for 18 hours for Colilert®-18 or 24 hours for Colilert®.
11.5.7. Remove the tray from the incubator and follow Section 13 to interpret the sample results. Any observed growth is unacceptable.
11.5.8. If growth is observed, repeat the procedure in 11.15. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer

11.6. Colilert® and Colilert®-18 Auto-fluorescence Check

11.6.1. Colilert® and Colilert®-18 will be checked for auto-fluorescence monthly or upon receipt of a new lot, which ever is sooner.
11.6.2. Add one packet of Colilert® or Colilert®-18 into a dilution bottle 100mL of SRGW.
11.6.3. Place the bottle on designated area (marked with tape) inside black box. Examine under 366-nm ultraviolet light for fluorescence.
11.6.4. Record the presence or absence of fluorescence, the new lot number, the manufacturer, the date received, and the analyst as appropriate. Fluorescence is unacceptable.

11.6.5. If the Colilert® or Colilert®-18 exhibits fluorescence, repeat the procedure. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer.

11.7. Colilert® and Colilert®-18 pH Check

11.7.1. The pH of Colilert® and Colilert®-18 is verified monthly or upon receipt of a new lot, which ever is sooner.
11.7.2. Add one packet of Colilert® or Colilert®-18 into a dilution bottle 100mL of SRGW.
11.7.3. Measure the pH.
11.7.4. Record the pH, the new lot number, the manufacturer, the date received, and the analyst as appropriate. The acceptable pH range is 7.0 – 7.6 SU.
11.7.5. If the pH is out of range, repeat the procedure in 11.7. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer.

11.8. Quanti-tray® Sterility

11.8.1. Upon receipt of each new lot of Quanti-trays® and Quanti-trays 2000®, a tray is analyzed to ensure sterility from bacterial contamination.
11.8.2. Aseptically transfer 100mL of sterile tryptic soy broth into the tray.
11.8.3. Seal the tray using the Quanti-tray® Sealer.
11.8.4.
11.8.5. Incubate the sealed Quanti-tray® and Quanti-trays 2000®, for 48 hours at 35.0°C ±0.5°C
11.8.6. Record the presence or absence of turbidity. Acceptable analysis should be free of turbidity.
11.8.7. If the tray containing TSB is turbid after 48 hours, repeat the procedures on another tray from the lot. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer.

11.9. Quanti-tray® Auto-fluorescence Check

11.9.1. Upon receipt of each new lot of Quanti-trays® and Quanti-trays 2000®, a tray is analyzed for auto-fluorescence.
11.9.2. Place a tray under 366-nm ultraviolet light in designated taped area in black box.
11.9.3. Recorded the presence or absence of fluorescence. Acceptable analysis shall not fluoresce.
11.9.4. If the tray fluoresces, one new tray from the same lot must be tested for fluorescence following procedure 11.9. If the reanalysis yields unacceptable results, notify the manufacturer and either discard or return the lot to the manufacturer.

11.10. Quanti-tray® Sealer Check

11.10.1. Once a month, the Quanti-tray® and Quanti-trays 2000®, Sealer is checked to ensure it is sealing properly.
11.10.2. Add four (4) to six (6) drops of food coloring to 100mL of RGW and pour into a Quanti-tray®.
11.10.3. Seal the tray using the Quanti-tray® and Quanti-trays 2000®, Sealer.
11.10.4. Acceptable analysis shall produce no leaks among the wells of the tray.
11.10.5. If leaks are present among the wells, repeat the procedure in 12.10. If the reanalysis fails, contact the manufacturer for the proper maintenance course of action. Utilize the backup Quanti-tray® Sealer or utilize other laboratory locations until maintenance is performed and an acceptable sealer check is produced.

11.11. Reagent Grade Water pH Check

11.11.1. The pH of the reagent grade water is measured monthly.
11.11.2. Measure an aliquot of sample on a bench top meter.
11.11.3. Acceptable pH shall be within the range of 5.5 – 7.5 SU.
11.11.4. If the pH is out of the acceptable range, repeat the procedure in 12.11. If reanalysis fails, contact the filtration system manufacturer for maintenance to resolve the problem.
11.11.5. The lot # of buffer used for calibration must be recorded.

11.12. Reagent Grade Water Conductivity Check

11.12.1. The conductivity of the reagent grade water will be measured monthly.
11.12.2. Be sure to rinse the conductivity probe twice with reagent grade water before taking the actual reading.
11.12.3. Acceptable measurements shall be < 2.0 µmhos/cm.
11.12.4. If the conductivity is > 2.0 µmhos/cm, repeat the procedure in 11.12. If reanalysis fails, contact the filtration system manufacturer for maintenance to resolve the problem.
11.12.5. Record the lot # of the standard on the benchsheet.

11.13. Reagent Grade Water Total Residual Chlorine Check

11.13.1. The total residual chlorine of the RGW is measured monthly.
11.13.2. Acceptable total residual chlorine results are < 0.02 mg/L.
11.13.3. If the chlorine is out of the acceptable range, repeat the measurement. If reanalysis fails, contact the filtration system manufacturer for maintenance to resolve the problem.
11.13.4. Record the lot # of the standard on the benchsheet.

11.14. Reagent Grade Water Heterotrophic Plate Count

11.14.1. Once a month, HPC's will be performed on the RGW from each of the three DI water stations: the Biology Lab, the Microbiology Lab and the Field Room (Organic-free water). These stations may vary between each laboratory location.
11.14.2. Follow the procedures for analyzing HPC in Aerobic/Heterotrophic Plate Count SOP.
11.14.3. Acceptable HPC results are < 500 CFU/mL.
11.14.4. If any of the stations produce unacceptable results, repeat that analysis by the procedure in 11.14. If reanalysis fails, contact the filtration system manufacturer for maintenance to resolve the problem.

11.15. 35°C Incubator Temperature Checks

11.15.1. The temperature of the 35°C Incubator must be checked twice daily, four hours apart, if possible, in order to assure the proper temperature is maintained. This is the incubator used in the analysis of all drinking water samples, and must be capable of maintaining this temperature. A temperature of 35± 0.5°C must be maintained.
11.15.2. A separate thermometer is on all three shelves within the incubator. Read the temperature of each thermometer.
11.15.3. If the temperature of the incubator is < 34.5 or >35.5°C, adjust the temperature. The temperature will then be monitored at the end of the day to assure proper temperature has been reached.
11.15.4. If the proper operating temperature is unable to be reached, contact a service company to service the incubator.

11.16. Microbiology Balance Check

11.16.1. The balance used to weigh all microbiology reagents is validated with ASTM Class 1 weights monthly.
11.16.2. Calibrate the balance as described Balance Operation and Calibration SOP.
11.16.3. The weights listed in Table 1 shall be measured. The acceptance range for each weight is listed below.

Table 1

Total Weight (g)	Acceptance Range (g)
1.0000	0.9995 – 1.0005
1.0020	1.0015 – 1.0025
5.0000	4.9995 – 5.0005
5.0020	5.0015 – 5.0025
10.0000	9.9995 – 10.0005
10.0020	10.0015 – 10.0025
50.00	49.95 – 50.05
50.10	50.05 – 50.15
100.00	99.95 – 100.05
100.10	100.05 – 100.15
150.00	149.95 – 150.05

11.16.4. If any of the weights are out of their acceptable ranges, the balance shall be re-calibrated and the analysis is repeated.
11.16.5. If any of the required weights are missing on the day of the monthly balance check, a replacement weight must be ordered immediately. Check the balance with the replacement weight upon arrival and with all scheduled monthly checks thereafter.

11.17. Conductivity Meter Quality Control Check
11.17.1. The accuracy of the conductivity meter is checked with a low conductivity calibration standard monthly.
11.17.2. Store the low conductivity calibration standard in the refrigerator. Allow the standard to equilibrate to room temperature (~20°C).
11.17.3. Calibrate the conductivity meter.
11.17.4. Rinse the conductivity probe twice with RGW and place in the low standard.
11.17.5. Acceptable standard for calibration must be < 15 µs/cm. Acceptable cell constant is 0.8 – 1.2.
11.17.6. If the conductivity calibration is unacceptable, repeat 11.16.1 - 11.16.5 using a new container of low conductivity standard. If the reanalysis fails, contact the instrument manufacturer for technical support.
11.17.7. Record the lot # of the standard on the benchsheet.

11.18. Autoclave Cleaning
11.18.1. Once a month, the autoclave is cleaned
11.18.2. The water chamber is drained and if necessary, the sides of the autoclaves are scrubbed with a sponge and RGW or distilled water until free of debris.
11.18.3. Clean autoclave door seals as needed to ensure they are free of caramelized media.
11.18.4. Document cleaning and any maintenance in the appropriate maintenance log.

11.19. Autoclave Timer Check
11.19.1. Quarterly, the autoclave timing cycle is verified to ensure acceptance.
11.19.2. Using a stopwatch, record the initial time the autoclave starts, the time the sterilization time starts, the time the sterilization time stops, and the time the entire cycle is completed.
11.19.3. Acceptable times are of 15 – 16 minutes for sterilization and no more than 45 minutes for a complete cycle.
11.19.4. If sterilization time is unacceptable, repeat the procedures in 11.19. If reanalysis fails, contact autoclave service contractor for technical support and/or maintenance.

11.20. Ultraviolet Lamp Reading
11.20.1. The ultraviolet light is tested with an UV meter to ensure that percent reduction in intensity is less than 70%. Quarterly checks are recommended.
11.20.2. Place UV meter in black box at location indicated with tape. Ensure that the scale face is vertical and the switch is in the "A" position. If black box is not utilized, be sure that the meter is placed at the same distance from lamp during each reading to ensure a consistent measurement.
11.20.3. Adjust the UV meter to zero by turning the screw located directly beneath the scale.
11.20.4. Plug the sensor cell directly into the receptacle on the top of the meter house.
11.20.5. Plug the extension cord into the receptacle with the red and black plugs aligned for proper polarity.
11.20.6. Turn on ultraviolet light and close the door to black box.
11.20.7. Record the result in uW/cm2 (where: Meter Reading X 100 = uW/cm2).
11.20.8. Calculate the percent reduction. Reduction greater than 70% is acceptable.
11.20.9. If the percent reduction is less than 70%, change the UV lamp bulb.

11.21. Ultraviolet Lamp Cleaning
11.21.1. Once a month, clean the ultraviolet lamp with a paper towel and ethanol such that it is free of debris.

11.22. Sanitizer Swab Test

11.22.1. Once a month, a sanitizer swab test is performed in the Microbiology Lab to ensure disinfectants are properly eliminating bacteria.
11.22.2. Wipe down one section of the Microbiology Lab counter with 50% Bleach, a second section of the Microbiology Lab counter with 2% Amphyl® and a third section of the Microbiology Lab counter with Lysol®. Be sure not to cross contaminate the disinfectants. (Note: If a particular disinfectant is not currently being used in the lab, this procedure is not applicable).
11.22.3. Once disinfectants have dried, swab each area with separate swabs.
11.22.4. Streak each swab onto separate pre-made plates of TSA or HPC labeled with the corresponding disinfectant name.
11.22.5. Incubate plates at 35.0°C+0.5°C for 48 ± 2 hours.
11.22.6. Remove the plates from the incubator and record the growth observed on the plates. Acceptable analysis will have no growth.
11.22.7. If there is any growth on any of the plates, reanalyze the disinfectant by the procedures of 11.22. If reanalysis fails, discard the failing disinfectant and use a new batch.

11.23. Air Plate Monitoring

11.23.1. Once a quarter, an air plate monitoring test is performed in the Microbiology Lab to ensure that the air does not contain bacterial contaminants. More frequent monitoring can be done as required by various site specific monitoring. Ex. Food in Louisville
11.23.2. Randomly pick two different locations in the Microbiology Lab.
11.23.3. Expose a pre-made, uncovered TSA or HPC plate to the air for 15 minutes in each location.
11.23.4. Replace the lids and incubate the plates 35.0°C± 0.5°C for 48 ± 2 hours
11.23.5. Remove the plates from the incubator and record the growth observed. Acceptable analysis yields no growth. For locations where food is not tested, acceptable reading is 15 or less colonies per plate.
11.23.6. If growth appears on either of the plates, replace the air filters.

11.24. Biological Indicator Sterility Check

11.24.1. Once a month, a biological indicator sterility check is performed to ensure the autoclave is sterilizing all contaminants.
11.24.2. Take a Biological Indicator and place in glass beakers.
11.24.3. Place beaker in the center of the autoclave. (Indicator may be placed directly in autoclave)
11.24.4. Autoclave with a 15 minute sterilization cycle at 121 -124°C /15 PSI.
11.24.5. Remove the beaker from the autoclave and place it in a 55.0°C – 60.0°C water bath or incubator along with one non-autoclaved indicator in a glass beaker as a control. Remove the beaker from the bath after 2 days (± 2 hrs from initiation time).
11.24.6. Remove the beaker from the water bath and record the results. The autoclaved biological indicators should be purple indicating sterility. The non-autoclaved control indicator should be yellow.
11.24.7. If autoclaved indicator is yellow after incubation, repeat the procedures in 11.24. If the reanalysis fails, perform a secondary test to determine whether the problem is with the indicators or the autoclave.
11.24.8. If the problem is with the autoclave, schedule maintenance.

11.25. Stock Culture QC

Bacteria *Genus species*	ATCC Strain	QC Purposes
Escherichia coli	25922	• Positive control for Colilert® and Colilert®-18 QC testing • Positive control for HPC's • Positive control for Fecal MPN's
Citrobacter freundii	8090	• Positive total coliform/non *E. coli* control for Colilert® and Colilert®-18 QC testing
Pseudomonas aeruginosa	27853	• Negative control for Colilert® and Colilert®-18 QC testing • Negative control for Fecal MPN's

11.26. Monthly Stock Culture Maintenance and Transfer:

11.26.1. Escherichia coli, Citrobacter freundii and Pseudomonas aeruginosa cultures shall be maintained in the Microbiology Lab at all times.

11.26.2. Aseptically transfer each bacterium from purchased swabs to new, sterile glass tubes with TSB. Label each tube with the name of the bacteria it contains, the date of preparation, and the analyst's initials.

11.26.3. Incubate tubes at 35.0°C+0.5°C for 24 hours (±1 hr). Screw top lids must remain loose to avoid pressure build-up during this time. The tubes should appear turbid with bacteria growth after this time.

11.26.4. Once a month, for five (5) consecutive months, each stock culture is aseptically transferred into a new sterile glass tube with a screw top lid containing TSB and incubated.

11.26.5. All expired and unused cultures must be autoclaved and discarded.

11.26.6. At the beginning of the sixth month, cultures are initiated from pure culture swab.

11.27. Quarterly Stock Culture Purity Check

11.27.1. Upon receipt of each new lot of cultures, a stock purity test will be performed. A quarterly check is optional.

11.27.2. Aseptically streak one loopful of each stock culture bacteria onto TSB agar or TSA plate.

11.27.3. Incubate plates at 35.0°C+0.5°C for 24 (± 1hr) hours.

11.27.4. Observe plates. Only a single colony type should be observed on each culture plate. Record observations in laboratory logbook.

11.27.5. Following manufacturer's instructions, a colony of each culture will be identified using Enterotube II.

11.27.6. Should culture not ID to expected species, immediately restart culture from pure culture swab obtained from outside manufacturer.

11.28. Temperature Distribution Studies

11.28.1. Annually, a study shall be performed on microbiology waterbaths and incubators to ensure an evenly distributed temperature.

11.28.1.1. Waterbaths

11.28.1.2. Place a NIST-certified thermometer in the center of the water bath and allow it to equilibrate to the temperature of interest.

11.28.1.3. Measure the temperature at each of the four corners of the water bath. Allow time for the thermometer to equilibrate between each reading.

11.28.1.4. The temperatures in each location shall be within 0.2°C of each other.

11.28.1.5. If the temperature variance is greater than 0.2°C, repeat the procedure of 11.28.1.1. If the reanalysis fails, contact the instrument manufacturer for technical support.

11.28.1.6. Incubators

11.28.1.7. Select one shelf to initiate study. Fill 5 media bottles with DI water and place in the four in the corners of the incubator or refrigerator shelf and place one in the middle of the shelf. Leave at least an inch clearance from the walls.

11.28.1.8. Allow the bottles of water to equilibrate to the temperature of the equipment being tested.

11.28.1.9. With the NIST traceable thermometer determine the temperature in each bottle.

11.28.1.10. Move the bottles to the next shelf and wait about one hour to allow to equilibrate.

11.28.1.11. Measure temperature per 11.28.1.9.

11.28.1.12. Continue this cycle until all shelves have been checked.

11.28.1.13. If the temperature variance is greater than 1.0°C, repeat the procedure. If the reanalysis fails, contact the instrument manufacturer for technical support.

11.29. Thermometer Calibration

11.29.1. All glass/mercury thermometers shall be annually verified, by a NIST-certified thermometer, to ensure accuracy.

11.29.2. All thermometers shall be calibrated at a temperature within 10 degrees of their use.

11.29.3. Use a beaker filled with water and ice on a stir plate for refrigerator thermometers and use a waterbath that is agitated to test incubator and waterbath thermometers.
11.29.4. To check verify autoclave thermometers, use a stirred oil sample heated to 121 ± 10°C.
11.29.5. Place NIST thermometer in close proximity to the glass/mercury thermometer being calibrated. The NIST thermometer should be immersed to the line marked on the thermometer.
11.29.6. Allow the temperatures to equilibrate.
11.29.7. Record the true temperature and the observed temperature in the appropriate spreadsheet. The correction factor will be calculated in the spreadsheet.
11.29.8. An acceptable correction factor must have less than 1.0°C of variability. Do not use thermometers outside this range for the drinking water program.
11.29.9. Label all thermometer with the date of calibration, date calibration is due, initials of person performing the calibration and the thermometer ID.
11.29.10. When labeling the thermometer is not feasible, such as with autoclave thermometers, post the label near the point of use. Also include the serial number to clarify the thermometer identification.

11.30. Total Organic Carbon, Silica and Ammonia on Reagent Grade Water

11.30.1. Once a month, the analysis of TOC, silica and NH3 on RGW shall be ensure that the levels are below the regulation limits.
11.30.2. Collect RGW in a plastic 250mL container preserved with sulfuric acid for NH3 analysis.
11.30.3. Collect RGW in 2 amber VOC vials preserved with sulfuric acid for TOC analysis.
11.30.4. Collect RGW in a plastic 250mL container for silica analysis.
11.30.5. Complete COC for the analysis of TOC, silica and NH3.
11.30.6. Relinquish the sample to operations for sample login and analysis.
11.30.7. Acceptable TOC results must be < 0.5 mg/L, silica below 0.05 mg/L and NH3 results < 0.1 mg/L.
11.30.8. If any results are out of range, repeat the procedures in 11.30. If reanalysis fails, contact the filtration system manufacturer for troubleshooting and/or maintenance.

11.31. Bacteria Suitability Test

11.31.1. Once a year, the RGW must be submitted to a certified subcontract laboratory to ensure that it is within the correct bacteriological ratio of growth.
11.31.2. Take a 100mL sterile Idexx bottle and rinse out sodium thiosulfate with organic-free reagent grade water.
11.31.3. Fill bottle to 100mL fill-line with reagent grade water.
11.31.4. Fill out proper chain of custody and relinquish sample to sample receiving.
11.31.5. The bacteria ratio of growth must be within the range of 0.8 – 3.0.
11.31.6. Send copy of results to the Commonwealth of Kentucky Microbiology Certification Authority.
11.31.7. If reagent grade water ratio of growth is out of range, the filtration system manufacturer will be notified so a proper technician can come out to resolve the problem.
11.31.8. Once technician has resolved problem, contact sub-contract laboratory to see if test can be performed a second time. (Test is usually only ran once a year.)
11.31.9. If able to resubmit sample, recollect sample and follow procedure 11.31.
11.31.10. If unable to resubmit sample, contact the Commonwealth of Kentucky Microbiology Certification Authority for further instructions.

11.32. Heavy Metals in DI Water

11.32.1. Once a year, the reagent grade water will be submitted to a certified laboratory to ensure that it is under the correct heavy metals reporting limit. Metals to be tested for include: chromium, nickel, cadmium, copper, lead, zinc, silver, arsenic, selenium, barium, and mercury.
11.32.2. Fill 250mL plastic bottle preserved nitric acid with RGW.
11.32.3. Fill out proper chain of custody and relinquish sample to sample receiving.
11.32.4. When results are received, file data in appropriate file in microbiology room.

11.32.5. Each metal contaminant must be < 0.05 mg/l; collectively, all metals must be < 0.1 mg/l.
11.32.6. Send copy of results to the Commonwealth of Kentucky Microbiology Certification Authority.
11.32.7. If any one of the metal contaminants is above the reporting limit, the filtration system manufacturer will be notified so a proper technician can come out to resolve the problem.
11.32.8. Once technician has resolved problem, repeat test beginning at 11.32.2.

11.33. UV Meter Calibration

11.33.1. Once a year the UV meter must be sent to the manufacturer to be calibrated.
11.33.2. A Scale must equal 600 µW/cm2. B Scale must equal 3000 µW/cm2.
11.33.3. Once meter is calibrated, give original certificate to QA/QC Officer and place copy of certificate in appropriate microbiology room file.

11.34. Autoclave Service

11.34.1. Once a year the autoclave must have a maintenance service provided.
11.34.2. Contact appropriate service technician. Autoclave temperature and pressure must be 119 - 124°C/15 PSI.

11.35. Inhibitory Residue Test

11.35.1. An inhibitory residue test must be performed to ensure the microbiology soap (if used) is not inhibiting the growth of bacteria. It is performed any time a new washing detergent is used or if glassware is cleaned with an acid wash. If glassware is not used during collection or analysis of drinking water sample, this test is not required.
11.35.2. Wash six 100 x 15 mm glass petri dishes as normal microbiology dishes: wash in neutral, non-inhibitory detergent, rinse once in tap water and twice in DI water. Label as Group A.
11.35.3. Wash six 100 x 15 mm glass petri dishes, however, rinse these an additional 10 times in DI water. Label as Group B.
11.35.4. Wash six 100 x 15 mm glass petri in neutral, non-inhibitory detergent and do not rinse at all. Label as Group C.
11.35.5. Autoclave all plates from Groups A, B and C with a 15 minute sterilization cycle.
11.35.6. Add 1.0 mL of polluted water yielding approximately 50 to 150 bacteria colonies. Obtain the polluted water by putting one loopful of bacteria (E. coli) into a 1000mL dilution series.
11.35.7. Prepare flask of HPC agar.
11.35.8. Add 12 – 15 mL of tempered agar onto each plate. Mix each plate slowly.
11.35.9. After the plates have solidified (approximately 10 minutes), invert and incubate plates at 35.0°C+0.5°C for 48+3 hours.
11.35.10. Interpretation of Results:
11.35.10.1. Difference in average number of colonies of less than 15% on plates of Group A, B and C indicate that the detergent has no toxicity or inhibitory effects.
11.35.10.2. Difference in colony count of greater than 15% or more between Groups A and B demonstrates that inhibitory residue is being left on dishes by the regular washing procedure.
11.35.10.3. Difference in average number of colonies of less than 15% between groups A and C indicate that the detergent has inhibitory properties that are eliminated during regular washing.
11.35.10.4. If there is a difference of greater that 15% among any of the groups, repeat test following procedures in 11.35.
11.35.11. If there is still a difference of greater than 15% among any of the groups, purchase a new detergent and perform inhibitory residue test on new product.

11.36. Analyst Blind Samples

11.36.1. Twice a year, all microbiology analysts will perform blind samples to demonstrate test performance capabilities.
11.36.2. In the first quarter of the year, the head Certification Authority will bring blind samples for all microbiology analysts to perform during the audit by the Commonwealth of Kentucky Microbiology Certification Authority.

11.36.3. Analysts will follow head Certification Authority's directions.
11.36.4. When completed, all analysts' results will be mailed to the Commonwealth of Kentucky Microbiology Certification Authority with the audit response.
11.36.5. Approximately six months from the time of the Certification Authority's blind samples, the MICROBAC QA/QC Officer or individuals assigned by QA Officer will make Blind PE Samples for all microbiology analysts.
11.36.6. If any analyst fails any blind sample, notify Commonwealth of Kentucky Microbiology Certification Authority for further instructions.

11.37. Performance Evaluation

11.37.1. Once a year the primary microbiology analyst will perform HPC, drinking water and wastewater Performance Evaluation samples to ensure test performance capabilities. Results will be mailed to the Commonwealth of Kentucky Microbiology Certification Authority.
11.37.2. If the analyst fails any of the tests, the Commonwealth of Kentucky Microbiology Certification Authority will notify MICROBAC with further instruction.

12. RESULTS, DATA ANALYSIS AND INTERPRETATION

12.1. Total Coliform Negative

12.1.1. After incubation, if the sample in the tray (if running quanti-tray) or bottle is clear, the sample is total coliform negative.
12.1.2. If the sample in any of the tray wells is yellow, but lighter than the yellow of the comparator, incubate the sample for up to 4 more hours (maximum of 28 total hours of incubation allowed) in the 35.0+0.5°C incubator. If all of the yellow wells are still lighter than the yellow of the comparator, the sample is total coliform negative.
12.1.3. From time to time, analysts may notice at the end of the 18 or 24 hour incubation period, samples appear turbid. This turbidity does not exhibit a yellow color. If this is observed, report sample as "Confluent Negative" and request a replacement sample from client.

12.2. Total Coliform Positive

12.2.1. After incubation, if the sample bottle or any of the wells (if quanti-tray is ran) is a yellow equal to or darker than the yellow of the comparator, the sample is total coliform positive. Record the number of small and large wells (if quanti-tray is ran) of this kind on the bench sheet use and IDEXX MPN Generator 3.2 to calculate the results.

12.3. E. coli Positive

12.3.1. If the sample (whole bottle or well) is total coliform positive and any of the wells fluoresce, the sample is E. coli positive. Record the number of small and large wells of this kind on the benchsheet and convert to the MPN using IDEXX MPN Generator 3.2.
12.3.2. Using a sterile pipet, pull approximately 0.5 mL of positive sample and place in a test tube.
12.3.3. Add 2-4 drops of Kovacs reagent to test tube.
12.3.4. If a pink/red ring forms around top of E. coli sample, the sample is confirmed to be E. coli positive. Red ring will develop in less than 30 seconds.
12.3.5. In LIMS. The below default results are as follows

Total coliforms (TC) → Absent
E. coli → NA

12.3.6. Be sure to manually update the results (both TC and E. coli) should a sample be positive for TC. A positive result must be entered as "Present" in LIMS.
12.3.7. If the pink/red ring does not form, the sample must still be reported as E. coli positive.

12.4. *E. coli* Negative

12.4.1. If the sample (either P/A bottle or individual well) is total coliform positive but does not fluoresce under the ultra-violet light, the sample is E. coli negative.

12.5. Reporting results

12.5.1. Results are reported in the following units:

Matrix	Significant	Wet / Dry Weight	Reporting Units

	Figures		
Total Coliform	3	N/A	MPN/100mL
E. coli	3	N/A	MPN/100mL
Total Coliform	--	N/A	P/A per 100mL
E. coli	--	N/A	P/A per 100mL

12.5.2. For MPN total coliform positive samples, count the number of yellow wells for each sample and record on the bench sheet. For presence/absence samples, record "P" or "A" on the bench sheet.

12.5.3. For E. coli, count the number of yellow fluorescing wells and record on the benchsheet.

13. MAJOR SOURCES OF UNCERTAINITY

13.1. Incubation time

13.2. Incubator temperature

14. TROUBLESHOOTING

Source	Description	Recommended Preventative Action
Quanti®-tray Sealer stopping	Occasionally the Sealer will stop in the middle of sealing a sample. The green light will turn off.	1. If the sample has not gone all the way into the sealer, pull the sample back out by using arrow button at the top of the machine. Wait for the green light to turn back on and place sample back in the sealer. 2. If the sample has gone all the way into the sealer, do not retrieve by pushing the arrow button. Wait until the green light turns back on and allow the sample to move through it.

15. WASTE MANAGEMENT

15.1.1. Microbac Laboratories and its employees must comply with all federal, state, and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

Waste Generated	Procedure for Handling and Disposal of Waste
Total coliform and *E. coli* positive sample trays	When ready to dispose of positive samples, place in red Biohazard bag to be autoclaved. Biohazardous waste will then be autoclaved at 121°C for a sterilization time of 30 minutes. It will then be disposed of in the regular MICROBAC waste.
Used and contaminated syringes	All used and contaminated syringes must be place in the Sharps Container. Once the Sharps Container is full, it will be permanently closed and autoclaved at 121°C for a sterilization time of 30 minutes. It will then be disposed of in the regular MICROBAC waste.

15.2. For further information on waste management, consult The Waste Management Manual for Laboratory Personnel and Less is Better: Laboratory Chemical Management for Waste Reduction, both available from the American Chemical Society's Department

16. REVISION HISTORY

16.1. 3/1/2010: Complete revision to include all QC requirements associated with the method.

16.2. 1/07/2010: Revised to add requirement for documentation, add pool holding time and completely revise thermometer calibration section.

APPENDIX B. PROTOCOL FOR POLYMERASE CHAIN REACTION AMPLIFICATION OF HOST-SPECIFIC BACTERIAL DNA SEQUENCES

MATERIALS

Water samples in sterile, EPA approved collection bottles, stored on ice.
UV-light sterilizer box
Vacuum aspiration manifold
100-mL graduated funnels
Filter forceps
Sterile phosphate buffered saline (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4; PBS)
Sterile 0.45-µm pore size membrane filters (Millipore, Billerica, MA)
Sterile 47-mm Petri plates (Millipore)
Membrane Enterococci Iron (mEI) agar (Difco, Detroit, MI)
Sterile 50-mL conical centrifuge tubes
Sterile 1.5-mL and 2.0-mL microcentrifuge tubes
Sterile 0.2-µm pore size Supor 200 PES membrane disc filters (Pall, Port Washington, NY)
Lysis buffer (20 m*M* EDTA, 400 m*M* NaCl, 750 m*M* sucrose, 50 m*M* Tris; pH 9.0)
Tryptic soy broth (TSB; Difco)
Tryptic soy agar (TSA; Difco) plate cultures of bacterial isolates
QIAamp DNA Mini Kit (Qiagen, Valencia, CA)
PowerWater® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA)
Lyse-N-Go™ reagent (Pierce Biotechnology, Rockford, IL)
ice buckets with ice
PCR primers on ice (Integrated DNA Technologies [IDT], Coralville, IA)
PCR Master Mix on ice (400 µ*M* of each dNTP; 50 units/mL *Taq* DNA polymerase; in Proprietary Reaction Buffer, pH 8.5, 3 m*M* $MgCl_2$; Promega, Madison, WI)
sterile reagent-grade, nuclease free, water (IDT)
sterile 0.2-mL PCR tubes
microcentrifuge tube rack
P2, P10 and P20 micropipetters, sterile white aerosol barrier micropipette tips, and sterile yellow micropipette tips
plastic discard beaker for used micropipette tips and microcentrifuge tubes
37°C water bath, 55°C water bath, 56°C water bath, and 95°C heat block
Vortex and Vortex adapter
microcentrifuge
thermocycler

PROCEDURE

Isolation of Enterococci *DNA*

1. Filter 100 mL of sample water through a sterile 0.45-µm pore size membrane filter (Millipore).
a. UV light sterilize the graduate funnels for a minimum of 2 min prior to use.
b. Ethanol dip and flame sterilize the filter forceps.

c. Transfer a membrane filter using the sterilized filter forceps from the membrane filter dispenser to the filter support mounted on the aspiration manifold.
d. Carefully seat a uv-light-sterilized graduate funnel onto the filter support.
e. Shake the water sample collection bottle vigorously, cut the security seal on the sample collection bottle, open the bottle, and pour the contents of the bottle into the graduated funnel.
f. Open the stop cock on the manifold stem, and turn on the vacuum pump.
g. Rinse the inside of the graduate funnel with sterile phosphate buffered saline (PBS).
h. Close the stop cock on the manifold stem, and turn off the vacuum pump.
2. Transfer the membrane filter using sterilized filter forceps from the filter support to a labeled, sterile 47-mm Petri plate (Millipore) containing 5 mL of sterile mEI agar (Difco).
3. Incubate the mEI agar plate culture at 41$_o$C for 48 hr (steps 1-3, USEPA, 2002).
4. After incubation, use sterilized filter forceps to transfer the membrane filter from the MEI agar plate to a sterile, 50-mL centrifuge tube containing 20 mL of sterile tryptic soy broth (TSB), and Vortex for 30 sec to mix.
5. Incubate the centrifuge tube at 41$_o$C for 3 hr to resuspend the bacterial cells in the TSB and partially enrich the culture. (steps 4 & 5, Scott, *et al.*, 2005)
6. Transfer 1 mL of resuspended cells to a sterile, labeled 1.5-mL microcentrifuge tube.
7. Pellet bacteria by centrifugation for 10 min at 5,000 x *g* (7,500 rpm).
8. Suspend the bacterial pellet in 180 µL of the lysozyme solution.
9. Incubate in a 37°C water bath for a minimum of 30 min.
10. Add 20 µL proteinase K and 200 µL Buffer AL. Vortex to mix.
11. Incubate at 56°C for 30 min and then for a further 15 min at 95°C.
12. Centrifuge for a few sec.
13. Add 200 µL 100% ethanol to the sample, and mix by pulse-Vortex for 15 sec. After mixing, briefly centrifuge the 1.5-mL microcentrifuge tube to remove drops from inside the lid.

NOTE: It is essential that the sample, Buffer AL, and ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form upon addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column.

14. Carefully apply the mixture from step 7 (including the precipitate) to the QIAamp Mini spin column (in a 2-mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6,000 x *g* (8,000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2-mL collection tube (provided), and discard the tube containing the filtrate.

NOTE: Close each spin column tightly to avoid aerosol formation during centrifugation.

15. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6,000 x *g* (8,000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2-mL collection tube, and discard the collection tube containing the filtrate.
16. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.
17. Place the QIAamp Mini spin column in a new 2-mL collection tube, and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

18. Place the QIAamp Mini spin column in a clean, labeled 1.5-mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µL Buffer AE. Incubate at room temperature for 1 min, and then centrifuge at 6,000 x *g* (8,000 rpm) for 1 min. This step elutes the DNA from the column into Buffer AE.
19. Carefully open the QIAamp Mini spin column and add an additional 200 µL Buffer AE. Incubate at room temperature for 5 min, and then centrifuge at 6,000 x *g* (8,000 rpm) for 1 min. After this step, 400 µL of DNA solution is present in the 1.5-mL microcentrifuge tube. (steps 6-19, Qiagen, 2010).
20. Assess the DNA solution spectrophotometrically to determine concentration and purity.
21. Store the DNA solution at -20°C until use.

Isolation of Bacterial DNA from Water Samples

1. Filter 1,000 mL of sample water through a sterile 0.2-µm pore size Supor 200 PES membrane disc filter (Pall), as described previously.
2. Using two sets of sterile filter forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward.
3. Insert the filter into the 5-mL PowerWater Bead Tube.
4. Add 1 mL of Solution PW1 (pre-warmed for 10 min in a 56°C water bath) to the PowerWater Bead Tube.
5. Secure the PowerWater Bead Tube horizontally to the Vortex adapter, and Vortex at maximum speed for 5 min.
6. Centrifuge the tubes 4,000 x *g* for 1 min at room temp.
7. Transfer all the supernatant, ~600-650 µL, to a clean 2-mL Collection Tube.
8. Centrifuge at 13,000 x *g* for 1 min.
9. Avoiding the pellet, transfer the supernatant to a clean 2-mL Collection Tube.
10. Add 200 µL of Solution PW2 and vortex briefly to mix. Incubate at 4°C for 5 min.
11. Centrifuge the tubes at 13,000 x *g* for 1 min.
12. Avoiding the pellet, transfer the supernatant to a clean 2-mL Collection Tube.
13. Add 650 µL of Solution PW3 (pre-warmed for 10 min in a 55°C water bath) and Vortex briefly to mix.
14. Load 650 µL of supernatant onto a Spin Filter and centrifuge at 13,000 x *g* for 1 min.
15. Discard the flow through and repeat until all the supernatant has been loaded onto the Spin Filter.
16. Place the Spin Filter basket into a clean 2-mL Collection Tube.
17. Shake to mix Solution PW4 before use. Add 650 µL of Solution PW4 and centrifuge at 13,000 x *g* for 1 min.
18. Discard the flow through and add 650 µL of Solution PW5 and centrifuge at 13,000 x *g* for 1 min.
19. Discard the flow through and centrifuge again at 13,000 x *g* for 2 min to remove residual wash.
20. Place the Spin Filter basket into a clean 2-mL Collection Tube.
21. Add 100 µL of Solution PW6 to the center of the white filter membrane.
22. Centrifuge at 13,000 x *g* for 1 min.
23. Discard the Spin Filter basket. The DNA is now ready for any downstream application (spectrophotometic assessment for concentration and purity; PCR); and can be stored at -20°C until use. (steps 2-23, MO BIO Laboratories, 2009).

Isolation of DNA from Bacterial Cultures

Add 7.5 µL of Lyse-N-Go™ reagent to a sterile, labeled PCR tube.

2. Using the same micropipet tip, scoop up a colony from a 24-hr TSA plate culture of the test bacterium and add it to the Lyse-N-Go™ reagent in the PCR tube. Avoid mixing.

3. Place the PCR tube in the thermocycler and run the program for Lyse-N-Go™ (total run time ~12 min):

- ▶ 65oC 30 sec
- ▶ 8oC 30 sec
- ▶ 65oC 90 sec
- ▶ 97oC 180 sec
- ▶ 8oC 60 sec
- ▶ 65oC 180 sec
- ▶ 97oC 60 sec
- ▶ 65oC 60 sec
- ▶ 80oC hold

4. Pulse the PCR tube in the microcentrifuge – this is referred to as Lyse-N-Go product, and contains the bacterial target (template) DNA. Use immediately in PCR.

PCR Amplification of the Enterococcus faecalis esp *Gene*

These methods adapted from Scott, *et al.* (2005).

1. Using sterile, nuclease free, aerosol barrier micropipette tips for PCR work.
2. For each PCR reaction add each of the following components, in the order indicated, to a new labeled PCR tube:
   - 2.0 µL (0.3 µM) forward primer (IDT; see Table 1 for specific primers)
   - 2.0 µL (0.3 µM) reverse primer (IDT; see Table 1 for specific primers)
   - µL extracted bacterial target (template) DNA
   - 7.5 µL sterile RNase free H_2O
   - 12.5 µL PCR Master Mix (Promega)

Table 1. PCR primers utilized for the detection of host-specific bacterial DNA sequences.

Gene	Bacterium	Primer	Sequence	Host Source
16S rRNA	*Bacteriodies-Prevotella*	HF183F Bac708R	5'-ATCATGATGTCACATGTCCG-3' 5'-CAATCGGAGTTCTTCGTG-3'	Human
16S rRNA	*Bacteriodies-Prevotella*	CF128F Bac708R	5'-CCAACYTTCCCGWTACTC-3' 5'-CAATCGGAGTTCTTCGTG-3'	Cattle
esp	*Enterococcus faecium*	espF espR	5'-TATGAAAGCAACAGCACAAGTT-3' 5'-ACGTCGAAAGTTCGATTTCC -3'	Human

3. For each set of PCRs conducted, several controls are also conducted:
A. Primer-only control:

- 2.0 µL (0.3 µM) forward primer (IDT; see Table 1 for specific primers)
- 2.0 µL (0.3 µM) reverse primer (IDT; see Table 1 for specific primers)
- 8.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

B. Target DNA template-only control:

- 1.0 µL extracted bacterial target (template) DNA
- 11.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

C. Non-target DNA control

- 2.0 µL (0.3 µM) forward primer (IDT; see Table 1 for specific primers)
- 2.0 µL (0.3 µM) reverse primer (IDT; see Table 1 for specific primers)
- 1.0 µL extracted *E. coli* (template) DNA (Lyse-N-Go product)
- 7.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

4. Place the PCR tubes into the thermocycler, and run the esp PCR program. The thermocycler conditions will be as follows:
Initial activation 2 min at 95 ° C
a. Denaturation 60 sec at 94 ° C
b. Annealing 60 sec at 58 ° C
c. Extension 60 sec at 72 ° C
35 cycles of steps 1-3
Final extension 7 min at 72 ° C
5. All PCR reaction products will be stored at -20 ° C until analysis by agarose gel electrophoresis.

PCR Amplification of the* Bacteriodies-Prevotella *16S rRNA Genes
These methods adapted from Bernhard and Field (2002a, 2002b).

1. Using sterile, nuclease free, aerosol barrier micropipette tips for PCR work.

2. For each PCR reaction add each of the following components, in the order indicated, to a new labeled PCR tube:

- 1.0 µL (10 µM) forward primer (IDT; see Table 1 for specific primers)
- 1.0 µL (10 µM) reverse primer (IDT; see Table 1 for specific primers)
- 1.0 µL extracted bacterial target (template) DNA
- 9.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

3. Repeat Step 2 using the PCR product of Step 2 as the target DNA.

4. For each set of PCRs conducted, several controls are also conducted:

a. Primer-only control:

- 1.0 µL (10 µM) forward primer (IDT; see Table 1 for specific primers)
- µL (10 µM) reverse primer (IDT; see Table 1 for specific primers)
- 10.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

b. Target DNA template-only control:
- 1.0 µL extracted bacterial target (template) DNA
- 11.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)
-

c. Non-target DNA control
- 1.0 µL (10 µM) forward primer (IDT; see Table 1 for specific primers)
- 1.0 µL (10 µM) reverse primer (IDT; see Table 1 for specific primers)
- 1.0 µL extracted *E. coli* (template) DNA
- 9.5 µL sterile RNase free H_2O
- 12.5 µL PCR Master Mix (Promega)

5. Place the PCR tubes into the thermocycler, and run the HF283 or CF128 PCR program. The thermocycler conditions will be as follows:
Initial activation 2 min at 95 ° C
a. Denaturation 30 sec at 94 ° C
b. Annealing 60 sec at 61 ° C (HF183F) or 58 ° C (CF128F)
c. Extension 60 sec at 72 ° C
25 cycles of steps 1-3
Final extension 6 min at 72 ° C
6. All PCR reaction products will be stored at -20ºC until analysis by agarose gel electrophoresis.

Protocol for the Analysis of PCR Products by Agarose Gel Electrophoresis

MATERIALS

PCR products
100-bp DNA ladder (New England BioLabs, Ipswich, MA)
6X gel loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 40% [w/v] sucrose)
Tris borate EDTA (1X TBE) buffer (90 m*M* Tris-borate, 2 m*M* EDTA, pH 8.3)
agarose (PCR grade, low EEO)
25-mL & 250-mL Erlenmeyer flasks
60ºC water bath
microcentrifuge tube rack
P20 micropipetter and sterile yellow micropipet tips
plastic discard beaker for pipet tips
Owl B2 EasyCast Mini Horitonal Gel System (Thermo Scientific, Rochester, NY)
20-tooth comb

electrophoresis power supply
staining tray and spatula
ethidium bromide stain (1.0 □g/mL)
UV Transilluminator and Photodocumentation System (Fotodyne, Hartland, WI)

PROCEDURE

These methods are adapted from Sambrook, *et al.* (1989).

1. Label a 250-mL Erlenmeyer flask "1.25% agarose" and add 80 mL of 1X TBE buffer to it.

2. Measure 1.0 g of agarose, add it to the TBE buffer and swirl to mix. Let stand for 5-10 minutes prior to heating to fully hydrate the agarose.

3. Place an inverted 25-mL Erlenmeyer flask in the mouth of the 250-mL flask and heat on a hotplate until the agarose completely dissolves. Remove the 250-mL flask from the hotplate periodically and swirl the contents.

SAFETY NOTE: Agarose can become superheated and boil over on a hotplate when swirled. Wear a thermal glove when removing the agarose and swirling heated flasks!

4. Place the agarose solution in a 55oC water bath until ready to use.

5. Prepare the gel tray for casting the agarose gel.

6. Pour the agarose solution into the gel tray to a depth of 4-5 mm. Place a 20-tooth comb in the appropriate slots in the casting tray.

7. Allow the gel to solidify on the bench for about 10 min. The gel will become translucent when solidified.

8. Rotate the gel tray in the electrophoresis chamber 90˚ so that the comb side of the gel is on the black (negative) electrode side of the electrophoresis chamber.

9. Add 1X TBE buffer to the electrophoresis chamber to a level about 0.5 cm above the center tray support.

10. Pour a small amount of 1X TBE around the comb and carefully remove the comb.

11. Add 5 µL of 6X gel loading buffer to each PCR reaction tube.

12. Set the P20 for 10 µL and load 10 µL of the 100-bp DNA ladder (Lad) to well #2 and #19. Next, set the P20 for 12 µL and load the remaining samples into the wells as illustrated in Fig. 1.

NOTE: Because of potential anomalies on the edge of agarose gels, the outer most wells are not used.

	1	2	3	4	5	6	7	8	...	18	19	20
	X	Lad	S1	S2	S3	S4	S5	S6	...	S16	Lad	X
Volume (µL)		10	12	12	12	12	12	12	12	12	10	

Figure 1. Lane assignments on the agarose gel.

13. Place the lid on the electrophoresis chamber and connect the leads to the power supply. Check to be sure that the wells are next to the negative (black) electrode so that the DNA will migrate toward the positive electrode (red).

14. Switch the power supply on, set the voltage at 80 V and electrophorese until the sample moves from the well into the gel. Increase the voltage to 120 V and continue the run until the bromophenol blue (the leading, darker dye) has migrated to about 3/4 of the way to the end of the gel.

15. When the electrophoresis is complete, turn the voltage down to the lowest level and switch the power supply off. Disconnect the leads from the power supply, and remove the lid from the electrophoresis chamber so that the gel can be removed.

16. Carefully remove the tray supporting the gel from the electrophoresis chamber and slide the gel into a labeled staining tray. Be careful as gels are slippery and can easily slide off the tray.

17. At the staining station, flood the gel with ethidium bromide (1 µg/mL) and stain for 10-15 minutes. Be sure to wear gloves when handling ethidium bromide.

SAFETY NOTE: Ethidium bromide is a mutagen and suspected carcinogen. Wear gloves, lab coat, and safety goggles, and work over absorbent plastic backed paper at all times when handling it.

18. Pour the ethidium bromide solution back into the storage bottle using the funnel provided. The staining solution can be reused 15-20 times.

19. Destain the gel for 5-10 minutes in distilled water. This will reduce background fluorescence, but this is usually not necessary unless very low amounts of DNA need to be visualized.

20. Use a plastic spatula to carefully lift the gel from the tray and slide it onto the UV transilluminator. Be careful to avoid trapping bubbles under the gel. The filter glass of transilluminators is very expensive and can be easily damaged if scratched.

SAFETY NOTE: UV light is hazardous and can damage your eyes. Never look at an unshielded UV light. Always view through a UV blocking shield or wear UV blocking safety goggles.

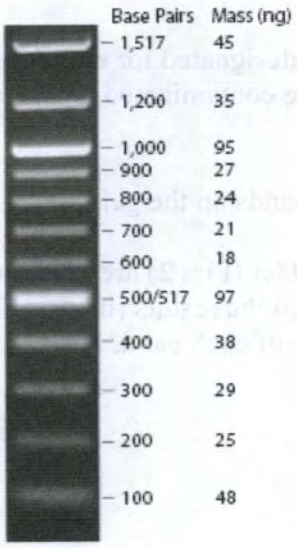
21. Use the FOTODYNE photo documentation system. Produce a thermal print of the gel for analysis. Additionally, save the image of the gel as a TIFF file on the PC. Be sure to back up the image file on a thumbdrive.

22. Discard the stained gel into a biohazard bag designated for ethidium bromide contaminated waste. Likewise, ethidium bromide contaminated gloves are also discarded in the same biohazard bag.

23. PCR product(s) will appear as one or more bands on the gel.

24. The size of the bands in the 100-bp DNA ladder (Fig. 2) are given in Table 2. Measure the distance migrated by each of the bands and record the results (in mm). Take the measurement from the bottom of each well to the leading edge of each band.

Figure 2. 100-bp DNA Ladder



Fragment #	Size (bp)
1	1,517
2	1,200
3	1,000
4	900
5	800
6	700
7	600
8	500/517
9	400
10	300
11	200
12	100

Table 2. The 100-bp DNA ladder.

25. Linear DNA fragments in an agarose gel migrate at a rate that is inversely proportional to the log of its molecular weight. When dealing with nucleic acids, size in base pairs is often substituted for molecular weight. Therefore, plotting the distance migrated versus the log of the fragment size will yield a straight line that can be used as a standard curve to determine the size of the other DNA molecules.

26. Using 2- cycle semi-log paper, plot the distance migrated by the bands of the 100-bp DNA ladder on the X-axis, and the size (in bp) on the log scale (Y-axis). Connect the data points with a straight line.

NOTE: the relation between distance and log bp will not be linear over the entire range.

27. Measure the distance of each PCR product(s) in each lane of the gel. Using these data and the standard curve, calculate the size of the PCR product(s).

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APPENDIX C. CHAIN OF CUSTODY FORM

Quality Assurance Project Plan Hancock Creek Watershed-Based Plan, Project 2011.
CFDA Number: Stream and Sampler Information

Site Identification:	Supervising Sampler:	Other samplers:
Date: Time:	Samples Collected: ☐ Bacteria (Sterile container) ☐ Nutrient and TSS Sample (500 mL acid washed container) ☐ Nutrient – acid treated	

Stream Conditions

Rain in the last 24 hours: ☐ 0 inch ☐ 0 – 1/2 inch ☐ ½ - 1 inch ☐ 1 - 2 inches ☐ 2 – 3 inches ☐ Other _____ Site source	Flow Rate (visual observation): ☐ Flood (over banks) ☐ Bank Full ☐ High Flow ☐ Normal ☐ Low ☐ Ponded (if ponded, samples will not be collected)
pH _____ Temp (C) _____ Conductivity _____ Average depth = (___+____+____+____+____+____+_____+____)/ ____ = ____ Average flow = (___+____+____+____+____+____+_____+____)/ ____ = ____	
Other Observations (smells, animals, land use changes, etc):	

Gage height (if applicable): _____

Sample Release (Sign and Record Date and Time)

Laboratory Drop Off	**Signature, Date, Time**
Microbiology Lab (Bacteria sample)	
Ecology Lab (Nutrient and TSS sample)	
Sampler (after dropping off all of the samples)	

Note: Physical parameter, velocity, depth, and channel width will be recorded in the field notebook of the sampler.

APPENDIX D. CALIBRATION PROCEDURES FOR DISSOLVED OXYGEN, TURBIDITY, CONDUCTIVITY, pH, and TEMPERATURE.

TEMPERATURE

The sondes utilize a thermistor of sintered metallic oxide that changes predictably in resistance with temperature variation. The algorithm for conversion of resistance to temperature is built into the sonde software, and accurate temperature readings in degrees Celsius, Kelvin, or Fahrenheit are provided automatically. No calibration or maintenance of the temperature sensor is required.

CONDUCTIVITY

The sondes utilize a cell with four pure nickel electrodes for the measurement of solution conductance. Two of the electrodes are current driven, and two are used to measure the voltage drop. The measured voltage drop is then converted into a conductance value in milli-Siemens (millimhos). To convert this value to a conductivity value in milli-Siemens per cm (mS/cm), the conductance is multiplied by the cell constant that has units of reciprocal cm (cm-1). The cell constant for the sonde conductivity cell is approximately 5.0/cm. For most applications, the cell constant is automatically determined (or confirmed) with each deployment of the system when the calibration procedure is followed.

MEASUREMENT AND CALIBRATION PRECAUTIONS

1. When filling the calibration vessel prior to performing the calibration procedure, make certain that the level of calibrant standard is high enough in the calibration cup or beaker to cover the entire conductivity cell.
2. After placing the sonde in the calibration solution, agitate the sonde to remove any bubbles in the conductivity cell.
3. During calibration, allow the sensors time to stabilize with regard to temperature (approximately 60 seconds) before proceeding with the calibration protocol. The readings after calibration are only as good as the calibration itself.
4. Perform sensor calibration at a temperature as close to 25oC as possible.

CALIBRATION STEPS

1. Place 200 mL of 10mS/cm conductivity standard into a clean, dry or pre-rinsed calibration cup.
2. Immerse the probe end of the sonde into the solution. Gently rotate the sonde up and down to remove any bubbles from the conductivity cell.
3. Allow at least one minute for temperature equilibration before proceeding.
4. From the Calibrate menu, select Conductivity to access the Conductivity calibration procedure and then 1-SpCond to access the specific conductance calibration procedure. Enter the calibration value of the standard you are using and press Enter.
5. Observe the readings under Specific Conductance or Conductivity and when they show no significant change for approximately 30 seconds, press enter. The screen will indicate that the calibration has been accepted and prompt you to press Enter again to return to the Calibrate menu.
6. Rinse the sonde in tap or purified water and dry the sonde.

pH

The sondes employ a field replaceable pH electrode for the determination of hydrogen ion concentration.

MEASUREMENT AND CALIBRATION PRECAUTIONS

1. When filling the calibration cup prior to performing the calibration procedure, make certain that the level
of calibrant buffers is high enough in the calibration/storage cup to cover at least ½ inch of the pH probe
and the temperature sensor of the 6560 probe.
2. Rinse the sensors with deionized water between changes of calibration buffer solutions.

CALIBRATION STEPS

1. Fill the calibration cup with 150 mL of pH 4 buffer standard, carefully immerse the probe end fo the sonde into the solution. Allow at least 1 minute for temperature equilibration before proceeding.
2. From the Calibrate menu, select 1SE1pH to accewss the pH calibration choices and then press 3- 3-Point.
3. Press Enter and input the value of the buffer at the prompt.
4. Press Enter and the current values of all enabled sensors will appear on the screen and change with time as they stabilize in the solution.
5. Observe readings under pH and when they show no significant change for approximately 30 seconds, press Enter. The display will indicate the calibration is accepted.
6. After the pH 4 calibration is complete, press Enter again, as instructed on the screen, to continue.
7. Rinse the sonde in water and dry the sonde before proceeding to the next step.
8. Repeat steps 1-7 to calibrate the pH probe for pH 7 and pH 10 buffer standards.
9. Rinse the sonde in water and dry. Thoroughly rinse and dry the calibration containers for future use.

TURBIDITY

Turbidity is the measurement of the content of suspended solids (cloudiness) in water and is typically determined by shining a light beam into the sample solution and then measuring the light that is scattered off of the particles which are present. For turbidity systems capable of field deployment, the usual light source is a light emitting diode (LED) which produces radiation in the near infrared region of the spectrum. The output of the sonde turbidity sensor is processed via the sonde software to provide readings in nephelometric turbidity units (NTUs).

MEASUREMENT AND CALIBRATION PRECAUTIONS

1. For best results, use only freshly prepared or purchased turbidity standards. Degradation of standards can occur on standing, particularly formazin prepared from dilution of concentrated suspensions such as Hach 4000 NTU standard.
2. If unusually high or jumpy readings are observed during the calibration protocol, it is likely that there are bubbles on the optics. Manually activating the wiper of the 6026 or 6136 from a computer or 650 MDS keypad removes these bubbles.

3. When calibrating the 6136 sensor, be aware of the fact that precautions must be taken to avoid interference of the bottom of the calibration vessel. Instructions for two methods of calibrating the 6136 sensor are provided in Section 2.6.1 of YSI Environmental Operation Manual manual. Unless these precautions are taken, field turbidity readings can exhibit an offset of approximately 1.5 NTU.

CALIBRATION STEPS

1. Fill the calibration cup with 200 mL of 0 NTU (clear deionized or distilled water) calibration standard. Immerse the sonde in the water.
2. Input the value 0 NTU at the prompt, and press Enter. The screen will display real-time readings that will allow you to determine when the readings have stabilized.
3. Activate the wiper 1-2 times by pressing 3-Clean Optics as shown on the screen, to remove any bubbles.
4. After stabilization is complete, press Enter to "confirm" the first calibration and then, as instructed, press Enter to continue.
5. Dry the sonde carefully and then place the sonde in the second turbidity standard (NTU 123) in the calibration cup.
6. Input the 123 NTU turbidity value and press Enter.
7. View the stabilization of the values on the screen in real-time.
8. After the readings have stabilized, press Enter to return to the calibrate menu.
9. Thoroughly rinse and dry the calibration cup for future use.

DISSOLVED OXYGEN

MEASUREMENT AND CALIBRATION PRECAUTIONS

1. Inspect the DO probe anodes; recondition using the 6035 reconditioning kit if they are not bright and shiny.
2. Install a new membrane, making sure that it is tightly stretched and wrinkle free. Warning: Replace the probe o-ring if it is loose or stretched out. If you remove the DO probe from the sonde, be sure to inspect the probe port and connectors for moisture. Remove any moisture droplets from the connector and thread areas. Verify that the probe is clean and dry then apply a small amount of synthetic grease to the o-ring before it is reassembled. Note: DO membranes will be slightly unstable during the first 3 to 6 hours after they are installed; it is strongly recommended that the final calibration of a DO sensor being used in "Unattended" studies takes place after this time period.
3. Go to the sonde's Report Menu and enable the "DO Charge". Now go to the Run Menu and start the sonde in the "Discrete Run" mode at a four-second rate. Allow the sonde to run (burn-in) for 15 minutes. Record the DO charge after about 5 minutes. The charge number must be between 25 and 75.
4. After the burn-in is complete, go to the sonde's Advanced Menu and confirm that the RS-232 Auto Sleep function is enabled.
5. Start the probe in the "Discrete Run" mode at a four-second rate and record the first 10 DO % numbers on paper. The numbers must start at a high number and drop with each four second sample. Example: 110, 105, 102, 101.5, 101.1, 101.0, 100.8, 100.4, 100.3, 100.1
It does not matter if the numbers do not reach 100%; it is only important that they have the same high to low trend. If you have a probe that starts at a low number and steadily climbs upwards, then the sensor has a problem and it must not be used.

Note: Initial power up can make the first two DO % samples read low; the first two samples can be disregarded.

CALIBRATION STEPS

1. Set the Auto Sleep RS-232 for the intended application: ON for UNATTENDED STUDIES and OFF for SAMPLING MEASUREMENTS.
2. Set the sonde into the calibration cup with approximately 1/8 inch of water. Do not engage the threads, and do not allow water to contact the membrane. You may also use the wet-towel method. The sonde must now sit in this saturated environment for at least 10 minutes before the DO calibration can begin. Warning: The sonde must be idle and not in the "Run" mode for 5 minutes prior to starting the DO calibration.
2. From the Calibrate menu, select Dissolved Oxy, then 1-DO% to access the DO percent calibration procedure.
3. Enter the current barometric pressure in mmHg.
4. Press Enter and the current values of all enabled sensors will appear on the screen and change with time as they stabilize.
5. Observe the readings under DO%. When they show no significant change for 30 seconds, press Enter.
6. The screen will indicate that the calibration has been accepted and prompt you to press Enter again to return to the Calibrate menu.
7. Rinse the sonde in water and dry the sonde.

Warning: Avoid having the DO probe membrane contact the calibration cup or sensor guard during transfers. Keep the DO probe in sight when removing or installing the sensor guard and cal cup.

REFERENCES

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