# Quality Assurance Plan for:
# **Winter Runoff of Surface Applied Animal Manure and Process Wastewater**

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**INTRODUCTION:** Land application of animal manure and process wastewater is among the documented sources of wet-weather water pollution causing degradation of streams, rivers and lakes in U.S. EPA Region 5. Recent documented cases have included ammonia toxicity or hypoxia as a cause of mortality to fish and other aquatic life, sedimentation, and pathogenic organisms that caused infections in humans. For example, Wisconsin recently documented 39 discharge incidents from land application of manure, one of which occurred during winter and wiped out ten years and $0.9 million of restoration work in the associated watershed (Donovan, 2005; Cain, 2005). In other major events in Wisconsin, improper winter land application killed the fish population of a lake and contaminated private drinking water wells (Vetrano, 2005; Donovan, 2005). Based in part on these incidents, the July 2005 draft strategic plan for the Region 5 Water Division included a new element providing that "we will improve EPA's winter land spreading guidance (Managing Manure Nutrients at Concentrated Animal Feeding Operations (EPA-821-B-04-006), available at http://cfpub.epa.gov/npdes/afo/info.cfm#manure) by sponsoring, or advocating OW- or ORD-sponsored research to fill data gaps on the quality of runoff from land on which certain manures have been applied." The National Center for Manure & Animal Waste Management, a consortium of 16 land grant universities supported by the United States Department of Agriculture, published Appendix L in the proceedings from its 2005 Symposium on the State of the Science for Animal Manure and Waste Management. The Region 5 Water Division uses the recommendations of this guidance document to evaluate the likely performance of state standards for land application of animal manure and process wastewater in the winter. States have developed or are developing these standards to meet Clean Water Act regulations for concentrated animal feeding operations (CAFOs). Current gaps in our knowledge limit the utility of this document as a means for evaluating state standards. Some of the gaps are identified in Appendix L, Table 5. The project proposed herein is intended to achieve this strategic element, thereby promoting progress toward the Region 5 Water Division's goals that all waters in Region 5 will support healthy aquatic biological communities and that designated swimming waters will be safe for swimming.

## SECTION 1.0, PROJECT DESCRIPTION AND OBJECTIVES

### *1.1 Purpose*

The primary purpose of the project is to protect fish and other aquatic life from organic matter, ammonia, and solids in animal manure and process wastewater. This will be accomplished by improving the scientific foundation for Appendix L in Managing Manure Nutrients at Concentrated Animal Feeding Operations (EPA-821-B-04-006). The secondary purpose of the project is to protect designated swimming waters from the pathogenic organisms in animal manure and process wastewater. Therefore, we also aim to improve understanding of the fate and transport of pathogenic organisms across unmanured setbacks and vegetative filter strips (VFSs) following application of animal manure to land. We will collect, analyze and evaluate concentrations of oxygen-demanding organic matter, ammonia, solids, and fecal bacteria at the edge of manured fields and down-slope of VFSs located at the USDA-ARS North Appalachian

Experimental Watershed (NAEW), near Coshocton, Ohio. We include plans to investigate the influence of rainfall and snow resulting in runoff events on movement of these pollutants across an unmanured setback and VFS. Non-critical molecular microbiological measurements will be used to support the critical (cultivation-based) fecal bacterial measures by characterizing the bacterial population with a greater degree of resolution than indicator approaches alone relative to specific pathogens, sources of fecal pollution, and other indicators of pathogenicity. These high resolution methods for microbial analysis may better pinpoint the effectiveness of the current and future management practices on the reduction of specific microorganism groups.

### *1.2 Site*

The NAEW is an agricultural, experimental watershed research facility located near Coshocton, Ohio, operated by the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS). Conservation tillage, filter strips, crop rotations, manure management, high runoff generating areas, reduced input management practices, and pasture management systems are all evaluated at the NAEW using small watersheds and monolith lysimeters (see Figure 1). Quantification of runoff and water quality risks through analysis of data and precipitation and weather investigations are also a component of the research. The NAEW is one of only two hydrologic stations worldwide with approximately 70 years of continuous data from rain gages, watershed flumes and weirs, and automated data collecting lysimeters. Along with soil and climatology data, these provide a long-term frame of reference which is essential in the evaluation of current experimental data.

The proposed work will be performed on several small experimental watersheds and on six VFS experimental plots. The experimental watersheds will be used for studying runoff over unmanured (but cropped) setbacks. The experimental plots for manure application are shown in Figures 2 and 3. These include watersheds 109, 118, 123, and 127, which will all be planted with corn and harvested. Watersheds 109, 118, and 123 are managed as no till, whereas watershed 127 is disked on contour in the spring. Watershed 113 and/or watershed 115 will be used as a no manure control. Watershed 111 will also be monitored as an alternative practice (no till corn, unmanured, and winter grazed rather than harvested). Runoff from the experimental watersheds will traverse over the unmanured setback (for manured fields), flow through an H-flume, and through a Coshocton wheel sampler that will capture a fraction of the flow for analysis.

The six experimental plots to be used for studying runoff over vegetative filter strips measure 12 m wide by either 140 m in length (3 plots with a 70 m long manure application area and 70 m long VFS) or 105 meters in length (3 plots with a 70 m long manure application area and 35 meter long VFS). The plots are constructed such that dustpan sample collectors (as described by Franklin et al., 2001, but modified to collect the 9/10 and 1/10 fraction of runoff) can be placed at the edge of the manured area capture runoff entering the VFSs, and cutoff walls placed on the down-slope end of the VFSs capture runoff in a gutter that extends the width of the plot (see Figures 4 and 5). Coshocton wheel samplers have been placed to capture flow-weighted composite samples from the VFSs during runoff events. The remainder of the runoff water flows overland to

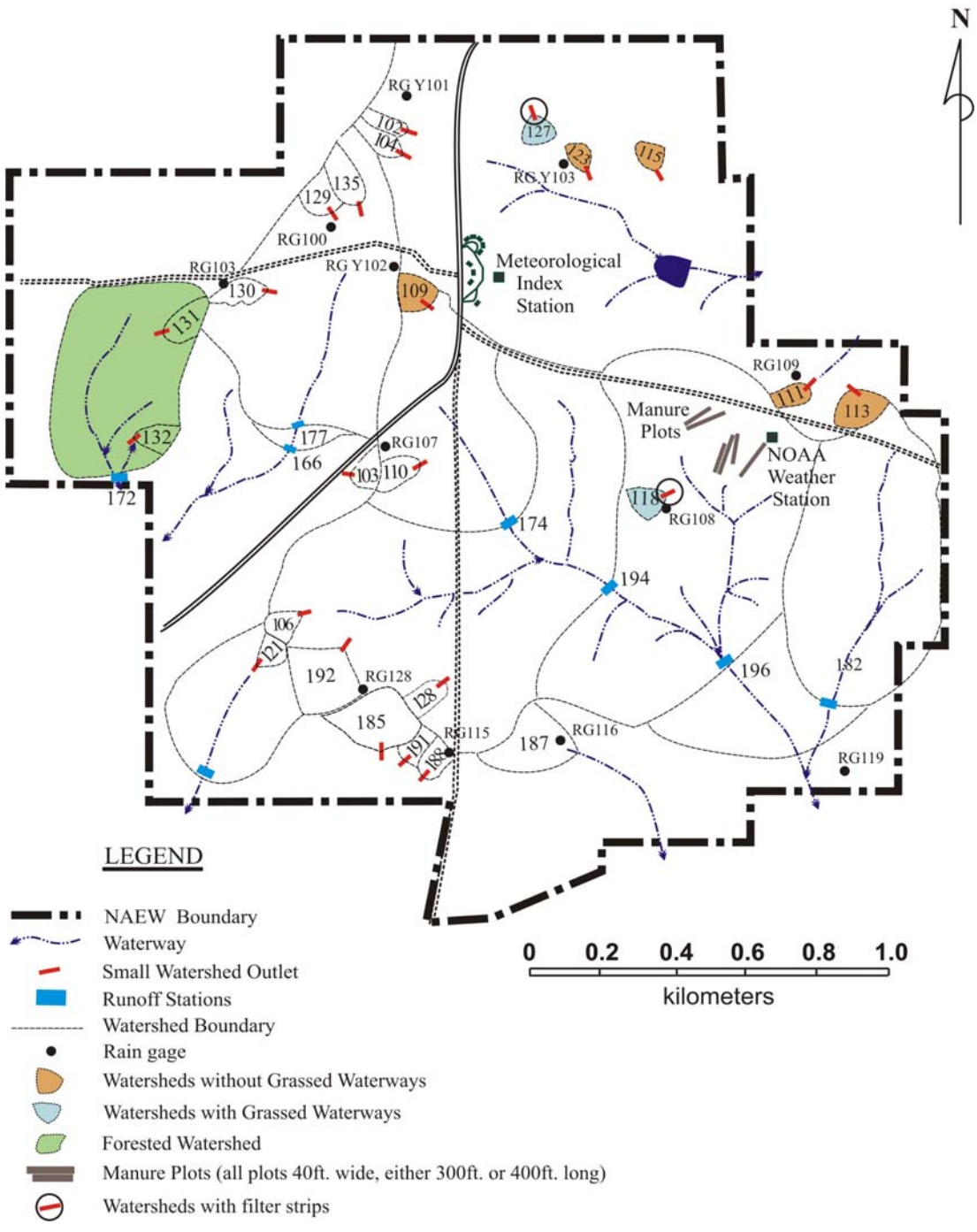


**Figure 1. North Appalachian Experimental Watershed (NAEW), 1047 acres of government-controlled land.[1]**

[1] From: U.S. Department of Agriculture, Agricultural Research Service and Soil Conservation Service, NAEW.

a receiving stream that is instrumented downstream the VFS experimental system with a weir to measure flow rate and automatic sampling equipment to measure water quality (runoff station 196, Figure 1). One or two additional sampling locations may be established at a later date upstream of runoff station 196, closer to the experimental VFS plots and watershed 118. Also instrumented with a weir is a forested watershed that may serve as a background "undisturbed" reference watershed (runoff station 172, Figure 1). Researchers with the USDA-ARS are planning to study the efficacy of VFSs for nutrient removal (ammonia-nitrogen, nitrate-nitrogen, and phosphate) in runoff from manured land, but have no specific plans to address oxygen-demanding organic materials or the movement of fecal pathogens through the study system. Consequently, the USEPA and ARS objectives are compatible. At present, there are no automatic sampling capabilities associated with the experimental VFS system except for Coshocton wheels.

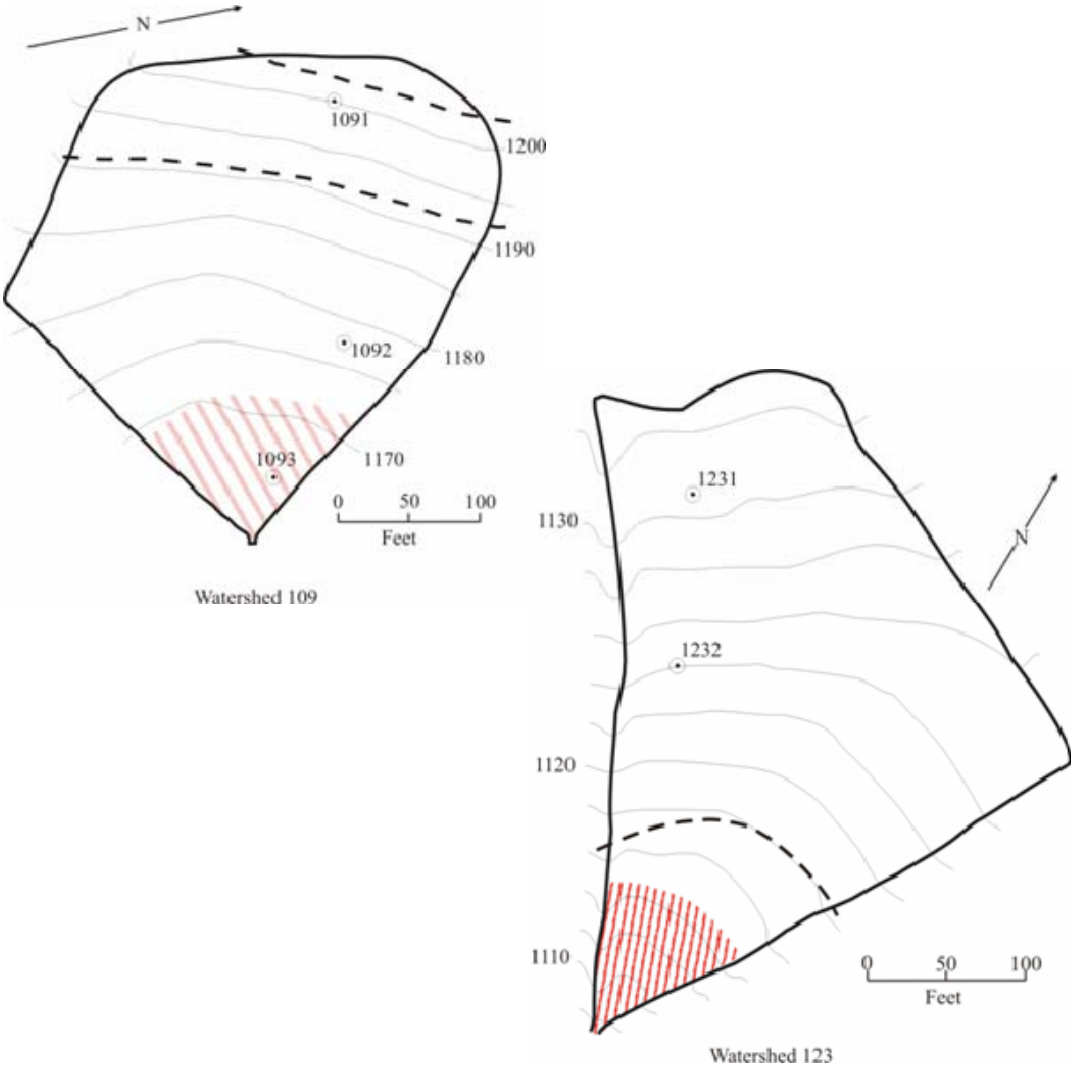


**Figure 2. Experimental (cropped) watersheds 109 and 123. These watersheds are managed as no-till corn and are planned for surface application of turkey manure at the nitrogen annual agronomic rate for corn for this study.**

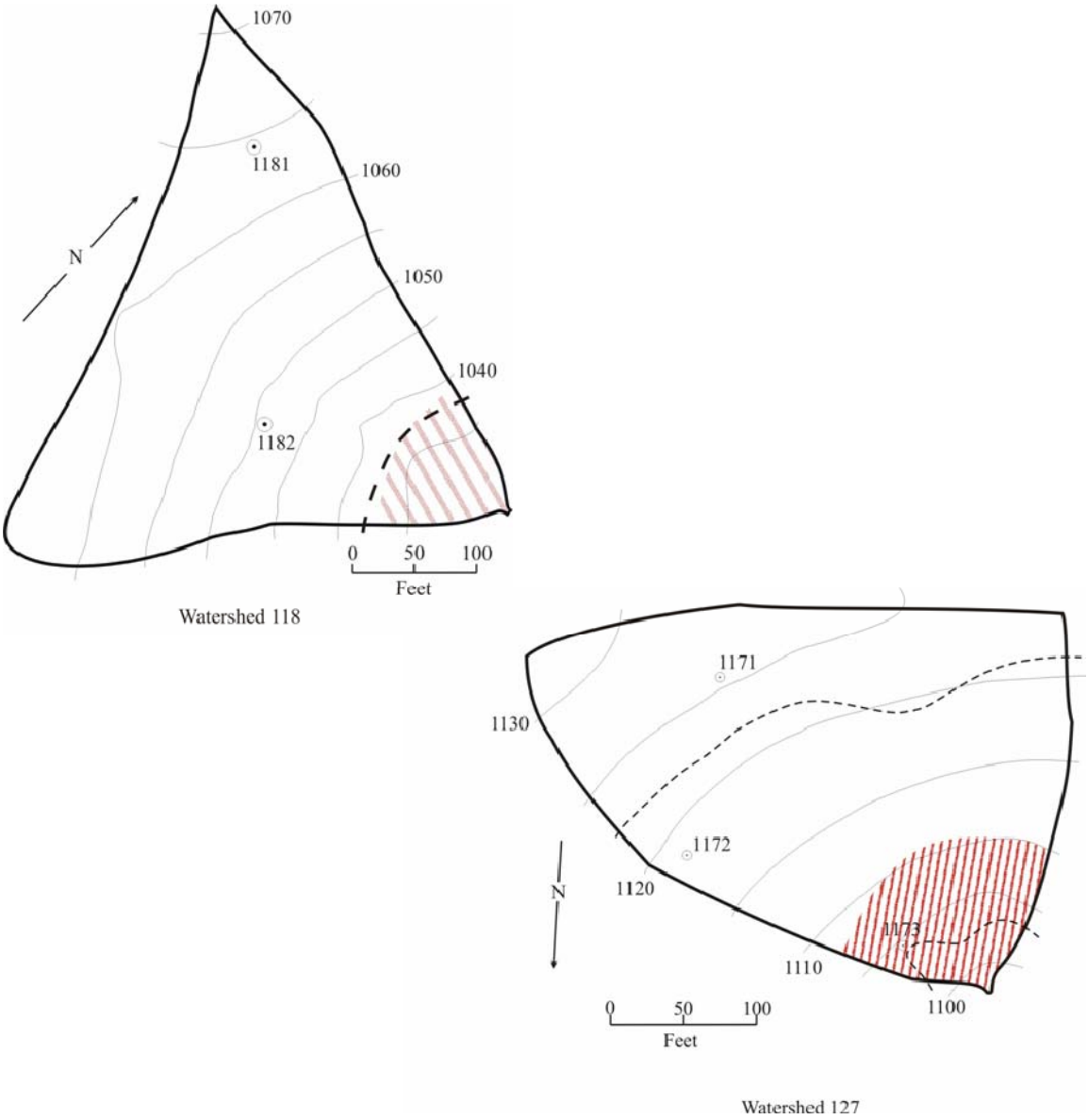


**Figure 3.** **Experimental (cropped) watershed 118 and 127. These watersheds are managed as no-till corn (WS118) and disked (on contour) corn (WS127) and are planned for surface application of swine manure at the nitrogen annual agronomic rate for corn for the study.**

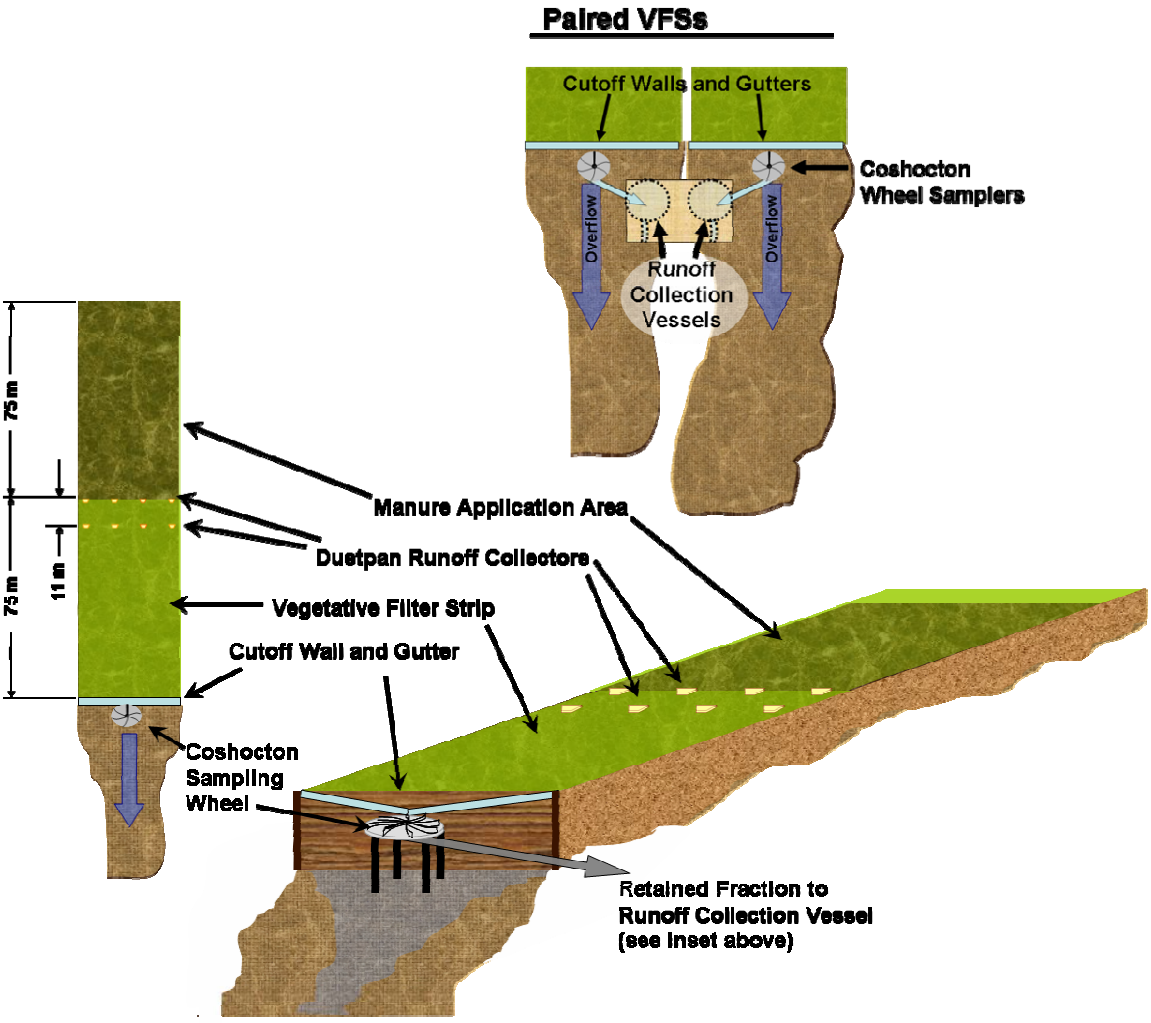


**Figure 4.** **Schematic layout of a 75 m VFS test plot at the NAEW (not to scale).**

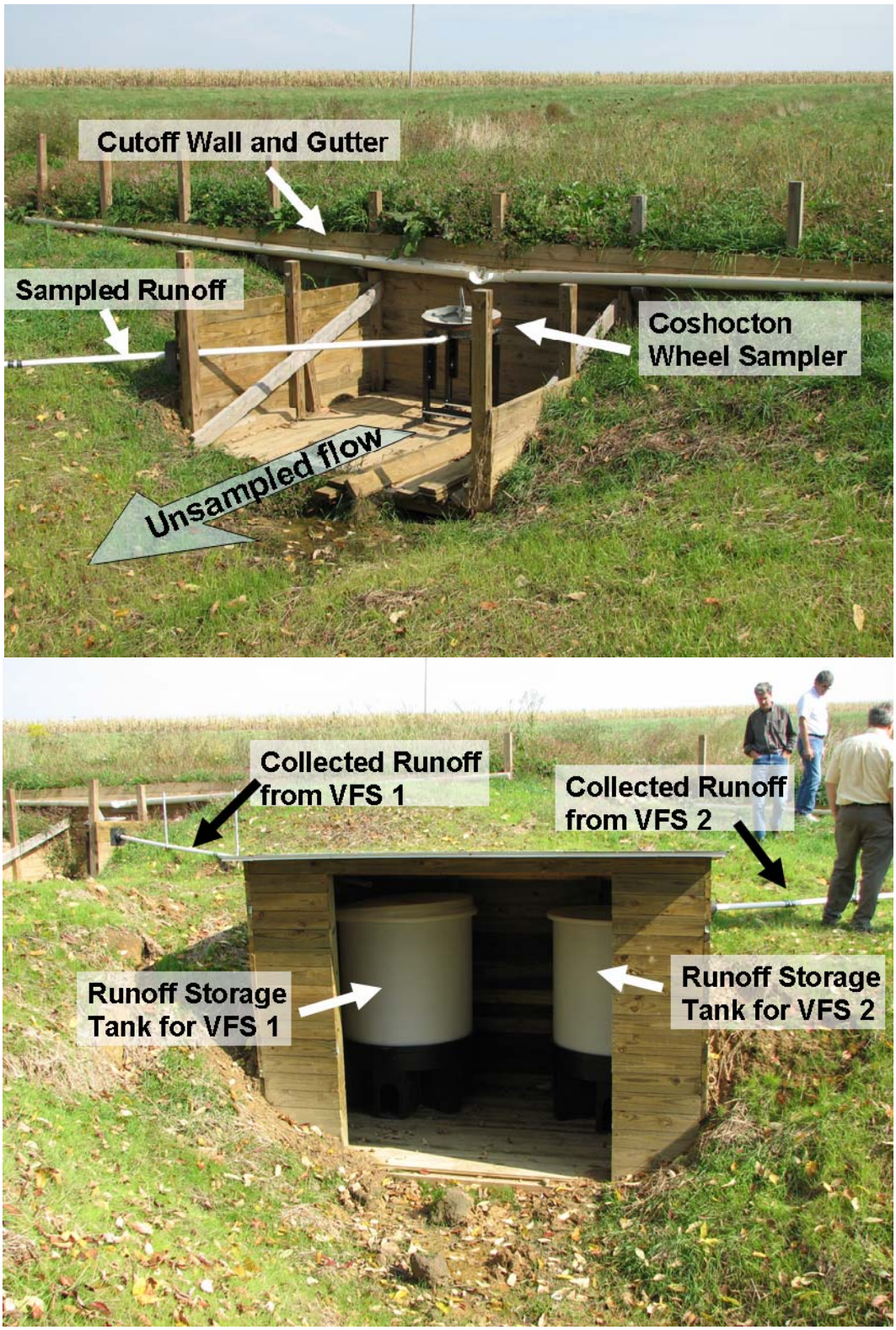


**Figure 5.** **Cutoff walls, Coshocton Wheel samplers, and runoff storage tanks for VFS 1 and 2 at the NAEW.**

### *1.3 Objectives*

The goal of this research is to improve the scientific foundation for guidance regarding the application of manure to land as described in Appendix L of Managing Manure Nutrients at Concentrated Animal Feeding Operations (EPA-821-B-04-006), and to evaluate the performance of VFS best management practices for limiting the movement of potentially pathogenic organisms from manured fields to nearby waters. We propose to address ***three primary objectives*** with critical (well-established) measurements at the NAEW. The ***first primary objective*** is to fill data gaps regarding the use of Appendix L, by (a) determining the "non-settleable" fraction of COD in several different animal wastes, yielding estimates for the variable "A" in equation 4; and (b) identifying "edge-of-field" COD values for winter surface application of cattle, swine, and turkey manure, which can be used to estimate COD in the discharge from an unmanured setback or VFS based on the calculated percent removal (the solution "E" of equation 4 of Appendix L).

The ***second primary objective*** is to determine if there is a statistically significant difference in runoff of COD following cattle, turkey, or swine manure application during the winter as compared to the spring. If a difference exists, we would like to better understand whether it is a result of changes in the loading rate at the upslope end of the setback or VFS ("edge-of-field" COD), the rate of depletion of COD within the setback or VFS (the fitted first-order reaction rate coefficient, variable "k", in Appendix L, Equation 4), or both. If the rate of depletion of COD within the setback or VFS varies considerably between winter and spring, we would also like to better understand the mechanisms for the change. For instance, although Equation 4 in Appendix L assumes that changes in COD within a VFS or setback are solely dependent on settling of non-soluble COD and degradation of soluble COD, the fitted first-order reaction rate coefficient for degradation of soluble COD (variable "k") will also implicitly include changes in COD due to infiltration into the soil profile, dilution (with rain water), and evapotranspiration. These factors can vary considerably between seasons, and may affect the overall best-fit first-order reaction rate coefficient within a setback or VFS. We hypothesize that reduced infiltration rates resulting from high antecedent soil moisture and/or frozen or snow-covered conditions in the winter relative to thawed and/or lower moisture conditions in the spring will lead to a much lower fitted first order reaction rate coefficient than currently used in Appendix L, equation 4. Use of an artificially large reaction rate could negatively impact the outcome of predictive modeling of COD discharge to surface waters whereby models under-predict actual discharge following manure application in the winter. Delivery of a more concentrated COD to the upslope of a setback or VFS from frozen or saturated fields may exacerbate the problem. Therefore, we plan to measure these factors to account for the various mass inputs and outputs that can affect the apparent depletion of COD within a setback and VFS in an attempt to construct a more useful model with more realistic first-order reaction rate constants for estimating treatment efficiency for winter manure applications. We will address these secondary sub-objectives to primary objective 2 with non-critical (novel) measurements.

The ***third primary objective*** is to determine if there is a statistically significant difference in runoff of *E. coli* and/or enterococci following cattle, swine, and turkey manure application during the winter compared to application during the spring. We plan to

compare both the reduction of *E. coli* and enterococci across the setback and VFSs and the effluent concentrations of these fecal indicator bacteria during spring and winter manure applications and runoff events. If resources allow, we will also compare runoff stations 172 and 196 to determine potential differences in bacteriological water quality that may be related to land use. As a sub-objective of primary objective 3, we will supplement the critical conventional indicator bacteria methods with non-critical (novel) high-resolution methods to better characterize the performance of the setback and VFSs under winter and spring conditions. High resolution methods will include identifying potential differences in antimicrobial resistance profiles, quantification of alternative (anaerobic) microbial indicators and specific pathogens using molecular microbial methods, microbial source tracking using non-library-based nucleic acid techniques, and detecting the presence of selected genetic virulence traits in *E. coli* and enterococci isolated from the applied manures and process wastewaters, crop-field and VFS runoff waters, and receiving waters that may serve as specific indicators of improvement in microbial water quality. Comparing methods for characterizing the microbial population will serve the long term goal of evaluating the performance of different management practices in reducing microbial pollutant movement into streams.

For objectives 1(b), 2, and 3, manure will be applied at (1) the nitrogen annual agronomic rate for corn on experimental watersheds 109, 118, 123, and 127, and (2) following NRCS guidelines for manure application (10 wet tons per acre for solid manures and 5000 gallons per acre for liquid manures and process wastewaters) on the VFS test plots. The annual agronomic N rate has been determined according to the Tri-State Fertility Guide, available at http://ohioline.osu.edu/e2567/index.html, and is shown in Table 1. If funds can be appropriated for an additional year or years of study, objectives 1(b), 2, and 3 will be replicated on the same plots with a manure type(s) different than cattle, swine, or turkey manure and/or a manure application rate equal to the phosphorus annual agronomic requirement for corn.

**Table 1. Planned manure applications on the NAEW watersheds for this study**

| Watershed | Treatment | Manure Application Rate **Kg N / Hectare** (lbs N / Acre) * | Watershed Size **Hectares** (Acres) † | Watershed Slope **(%)** § |
|---|---|---|---|---|
| WS 118 | Swine Manure | **181.3** (160) | **0.79** (1.96) | 11 |
| WS 127 | Swine Manure | **181.3** (160) | **0.67** (1.65) | 10 |
| WS 109 | Turkey Manure | **181.3** (160) | **0.68** (1.69) | 12 |
| WS 123 | Turkey Manure | **181.3** (160) | **0.55** (1.37) | 6 |
| WS 113 | No Manure | – | **0.59** (1.46) | 10 |
| WS 115 | No Manure | – | **0.65** (1.61) | 8 |

***** Annual nitrogen agronomic rate for corn as determined using the "Tri-State Fertilizer Recommendations for Corn, Soybeans, Wheat and Alfalfa" Bulletin for a potential yield of 140 bu/As corn.

† Size of the entire watershed; manure will not be applied within 100 ft of the flume.

§ Average slope approximated from contour maps

## SECTION 2.0, PROJECT ORGANIZATION

### *2.1 Key Contacts*

#### 2.1.1 U.S. Environmental Protection Agency, Office of Research and Development

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## *2.2 QA Manager*

Scott Jacobs (513-569-7223) is the QA Manager for the U.S. EPA, ORD, NRMRL, LRPCD and is responsible for review and approval of the QAPP.

## *2.3 Responsibilities*

James Bonta, Lloyd Owens, and Martin Shipitalo of the USDA-ARS will be responsible for installation of any pertinent sampling equipment and for collection, preservation, and shipment of samples, maintenance of field sampling records, and provision of a copy of all field records to Drs. Rogers and Haines following sample collection. They will be responsible for planning and coordinating field sampling events and measuring total and nonsettleable COD in the runoff waters using Standard Methods for the Examination of Water and Wastewater. As such, they will also be responsible for providing all necessary equipment and reagents for COD analysis. When runoff events are such that samples for bacteriological water quality cannot be received in the AWBERC facility within 48 hours of sample collection (samples collected between Thursday evening and Sunday morning), *E. coli* and enterococci will be measured by USDA-ARS staff at the NAEW facility.

Shane Rogers and John Haines of the USEPA-NRMRL will be responsible for training NAEW staff in proper sample collection, analysis, and preservation techniques (if necessary) and will provide all pertinent sample collection bottles, reagents, tubes and buffers, labels, and sample record logs for analyses to be carried out at the USEPA AWBERC facility in Cincinnati, OH. Drs. Rogers and Haines will also be responsible for receiving samples from the research site and measuring *E. coli*, enterococci, and antimicrobial resistance in the laboratory. Further, they will be responsible for measuring total and nonsettleable COD in the various manures and process wastewaters using Standard Methods for the Examination of Water and Wastewater. Shane Rogers will be responsible for development and oversight in the application of all molecular microbiological methods. All investigators will share responsibility for data management, analysis, and interpretation.

## SECTION 3.0, EXPERIMENTAL APPROACH

### *3.1a General Approach – Primary Objectives*

There are three primary objectives of the study which will be addressed with critical measurements. Primary objectives 2 and 3 will be further explored with several non-critical sub-objectives. The approach for each is as follows:

***Primary Objective 1:*** *Fill in data gaps regarding the use of Appendix L.*

***Primary Sub-objective 1a (critical measurement):*** *Determine the total and "non-settleable fraction" of the chemical oxygen demand (COD) and/or biological oxygen demand ($BOD_u$) of several animal manures and process wastewaters which may include, in order of priority:*

*(a) manure from mature dairy cows,*
*(b) swine manure,*
*(c) manure from egg-laying chickens,*
*(d) turkey manure,*
*(e) manure from beef cattle,*
*(f) manure from broiler chickens,*
*(g) egg wash process wastewater,*
*(h) cattle open lot process wastewater, and*
*(i) process wastewater from feed storage.*

Approach: Collect manure and process wastewater samples representative of fresh or minimally stored wastes for the sources described above and analyze for total and nonsettleable COD (and $BOD_u$ on selected samples) using Standard Methods for the Examination of Water and Wastewater. The nonsettleable $COD/BOD_u$ will be measured from the supernatant following settling for 1 hour in an Imhoff cone or equivalent, as suggested by Fischer and Symons (1944) and Tchobanoglous and Schroeder (1985). The settleable $COD/BOD_u$ will be assumed equal to the total $COD/BOD_u$ less the nonsettleable $COD/BOD_u$. The "non-settleable fraction" will be calculated by dividing the nonsettleable

$COD/BOD_u$ by the total $COD/BOD_u$. The materials described in (c) through (f) will be solids, and will be tested for nonsettleable $COD/BOD_u$ by preparing dilutions of solid manures with sterile water prior to settling. Preferably, three or more large CAFO sources for each waste type will be used and the average values reported. However, the availability of potential CAFO collaborators will determine which of the above waste types will be analyzed and the final number of sources for each waste type.

PO Hypothesis 1a: This is a data-driven objective rather than hypothesis-driven.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.).

*Expected measurement range:* $COD/BOD_u$ in the different process wastewaters and animal wastes are expected to range from 100 mg/L – 100,000 mg/L.

***Primary Sub-objective 1b (critical measurement):*** *Measure the "edge-of-field" (unmanured setback or VFS influent) total COD and "non-settleable" COD for winter application of manure to obtain a value for use in Step 5 in Appendix L and thereby estimate COD in the discharge from an unmanured setback or a VFS based on calculated percent removal (E in Equation 4, Appendix L).*

Approach: Collect runoff samples from the dustpan sample collectors following application of manure to frozen ground in the winter (target dates of December 21 and March 21, pending weather uncertainties) and test for total COD and nonsettleable COD using Standard Methods for the Examination of Water and Wastewater and the methods described for non-settleable solids defined in Primary Sub-Objective 1a. For the purposes of this study, frozen ground will be quantified by using an array of soil temperature sensors with depth, with increasing spacing with depth. Runoff samples will be taken during several discharge events that will include the first event following manure application. Swine and/or turkey manure will be surface-applied to the experimental (cropped) watersheds at a rate equal to the nitrogen annual agronomic requirements for corn. Edge-of-field runoff from these small watersheds will be collected in dustpan sample collectors at the edge of the manured areas, prior to the 30 m unmanured setbacks. Cattle manure will be surface applied to the VFS test plots at a rate equal to NRCS recommendations. Edge-of-field runoff from these plots will be collected in dustpan sample collectors placed at the edge of the manured area, upslope of the vegetative buffers. The total COD (minus background COD determined from unmanured control plots) will be used in Step 5 of Appendix L.

PO Hypothesis 1b: This is a data-driven objective rather than hypothesis-driven.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.).

*Expected measurement range:* Total and nonsettleable COD at the "edge-of-field" is expected to range from 100 mg/L – 100,000 mg/L.

***Primary Objective 2:*** *Determine if there is a statistically significant difference in runoff of COD from manure-amended fields in the spring and winter.*

> ***Primary Sub-objective 2a (critical measurement):*** *Determine if there is a statistically significant difference in the "edge of field"total and/or nonsettleable COD in runoff following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). For purposes of this study, the "edge-of field" is defined as the line that divides the manure application area and the upslope beginning of the VFS or unmanured setback.*
>
> Approach: Collect runoff samples from the dustpan sample collectors following surface application of manure to frozen ground in the winter (target dates of December 21 and March 21, pending weather uncertainties) and following an equivalent application to thawed ground in the spring (expected to be between the first day after March 21 during which soil can handle manure hauling equipment without undesirable compaction and the last day during which the crop can be planted while achieving a realistic yield, but pending weather uncertainties) and test for total and nonsettleable COD (and $BOD_u$ on selected samples) using Standard Methods for the Examination of Water and Wastewater and the methods described for non-settleable solids defined in Primary Sub-Objective 1a. Runoff samples will be taken during several discharge events that will include the first event following manure application in each season. Swine and/or turkey manure will be surface-applied to the experimental (cropped) watersheds at a rate equal to the nitrogen annual agronomic requirements for corn. Edge-of-field runoff from these small watersheds will be collected in dustpan sample collectors at the edge of the manured areas, prior to the 30 m unmanured setbacks. Cattle manure will be surface applied to the VFS test plots at a rate equal to NRCS recommendations. Edge-of-field runoff from these plots will be collected in dustpan sample collectors placed at the edge of the manured area, prior to the vegetative buffers. Manure applications will occur in subsequent years with the winter application occurring in the first year of study and the spring application occurring in the second year of study. Frozen ground will be defined as in sub-objective 1b. Inferential statistics will be used to test for differences in runoff of total and/or nonsettleable COD at the edge-of-field due to surface application to frozen or thawed ground, and are described in detail below.
>
> PO Hypothesis 2a-1: The "edge-of-field" *total* COD in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Hypothesis 2a-2: The "edge-of-field" *nonsettleable* COD in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Null Hypothesis 2a-1: The "edge-of-field" *total* COD in runoff does not differ between winter and spring manure applications.

PO Null Hypothesis 2a-2: The "edge-of-field" *nonsettleable* COD in runoff does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the "edge-of-field" COD in runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Total and soluble COD at the "edge-of-field" is expected to range from 100 mg/L – 100,000 mg/L.

***Primary Sub-objective 2b (critical measurement):*** *Determine if there is a statistically significant difference in the total and/or nonsettleable COD in runoff from unmanured setbacks or VFSs following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). For the purposes of this study, runoff from the 30 m unmanured setbacks will be defined as that which is captured in the Coshocton Wheel samplers installed in the runoff collection stations fitted at the down-slope end of the experimental (cropped) watersheds. Runoff from the 35 and 70 m VFSs will be defined as that which is captured in the Coshocton Wheel samplers installed at the cutoff wall of the experimental VFSs. Runoff from an 11 m VFS will be defined as that which is captured in dustpan sample collectors placed 11 m down-slope the edge-of-field in the VFS test plots.*

Approach: Collect runoff samples from the Coshocton wheel samplers at the down-slope end of the unmanured setbacks and VFSs and from dustpan sample collectors located at 11 m down-slope from the edge-of-field in the VFSs following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring (as defined in Sub-objective 2a, above), and test for total and nonsettleable COD (and $BOD_u$ on selected samples) as described in sub-objective 2a. Manure application and runoff samples will coincide with those of sub-objective 2a. Inferential statistics will be used to test for differences in total and/or nonsettleable COD in the unmanured setback or VFS runoff due to surface application to frozen or thawed ground, and are described in detail below.

PO Hypothesis 2b-1: The *total* COD in runoff collected down-slope of unmanured setbacks and VFSs resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Hypothesis 2b-2: The *nonsettleable COD* in runoff collected down-slope of unmanured setbacks and VFSs resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Null Hypothesis 2b-1: The *total* COD in runoff collected down-slope of unmanured setbacks and VFSs does not differ between winter and spring manure applications.

PO Null Hypothesis 2b-2: The *nonsettleable* COD in runoff collected down-slope of unmanured setbacks and VFSs does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the COD in setback and VFS runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Total and nonsettleable COD in runoff from the setback and VFS is expected to range from 100 mg/L – 100,000 mg/L.

***Primary Objective 3:*** *Determine if there is a statistically significant difference in runoff of potentially pathogenic bacteria from manure-amended fields in the spring and winter.*

***Primary Sub-objective 3a (critical measurement):*** *Determine if there is a statistically significant difference in the "edge of field" concentration of E. coli and/or enterococci in runoff following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). Manure application and runoff sampling will coincide with that described in Sub-objective 2a.*

Approach: Collect runoff samples from the dustpan sample collectors following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring and test for *E. coli* and enterococci using Idexx defined substrate technology. Manure application and runoff samples will coincide with those of sub-objective 2a. Inferential statistics

will be used to test for differences in runoff of *E. coli* and enterococci at the edge-of-field and are described in detail below.

PO Hypothesis 3a-1: The "edge-of-field" concentration of *E. coli* in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Hypothesis 3a-2: The "edge-of-field" concentration of enterococci in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

PO Null Hypothesis 3a-1: The "edge-of-field" concentration of *E. coli* in runoff does not differ between winter and spring manure applications.

PO Null Hypothesis 3a-2: The "edge-of-field" concentration of enterococci in runoff does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the "edge-of-field" *E. coli* and/or enterococci in runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range: E. coli* and enterococci at the "edge-of-field" are expected to range from 100 MPN/100 mL – 10,000,000 MPN/100 mL.

***Primary Sub-objective 3b (critical measurement):*** *Determine if there is a statistically significant difference in the concentrations of E. coli and/or enterococci in runoff from unmanured setbacks or VFSs following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). Manure application and runoff sampling will coincide with that described in Sub-objective 2b.*

Approach: Collect runoff samples from the Coshocton wheel samplers at the down-slope end of the unmanured setbacks and VFSs and from dustpan sample collectors located at 11 m down-slope from the edge-of-field in the VFSs following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring (as defined in Sub-objective 2a, above), and test for *E. coli* and enterococci using Idexx defined substrate technology. Manure application and runoff samples will coincide with those of sub-objective 2b. Inferential statistics will be used to test for differences

in *E. coli* and/or enterococci in the unmanured setback or VFS runoff due to surface application to frozen or thawed ground, and are described in detail below.

PO Hypothesis 3b-1: The concentration of *E. coli* in unmanured setback and VFS runoff resulting from winter manure application is statistically significantly different than that resulting from spring manure application.

PO Hypothesis 3b-2: The concentration of enterococci in unmanured setback and VFS runoff resulting from winter manure application is statistically significantly different than that resulting from spring manure application.

PO Null Hypothesis 3b-1: The concentration of *E. coli* in unmanured setback and VFS runoff does not differ between winter and spring manure applications.

PO Null Hypothesis 3b-2: The concentration of enterococci in unmanured setback and VFS runoff does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the *E. coli* and/or enterococci in unmanured setback or VFS runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range: E. coli* and enterococci discharging from the unmanured setback and VFS are expected to range from 100 MPN/100 mL – 1,000,000 MPN/100 mL.

### *3.1b General Approach – Secondary Objectives*

The secondary objectives of the study are to be addressed with non-critical (novel) measurements and include: (1) supplementing conventional coliform analysis methods with higher resolution molecular microbiological methods for characterizing the microbial community associated with runoff from manure-treated land; and (2) collecting physical information on the soil profile under frozen and thawed conditions to improve our understanding of the fate and transport of pollutants across unmanured setbacks and VFSs. Addressing infiltration will require the use of a weather monitoring station and soil temperature and moisture probes and/or other methods for measuring infiltration into the soil profile. Supplementing conventional coliform analysis methods may involve the use of advanced molecular microbiological methods, some of which will be optimized for our particular sample matrices during the course of this study. It is unclear whether the populations of the specific pathogens will be large enough to allow for testing of

statistically significant differences in any of these objectives. The approach for each secondary objective is as follows:

***Secondary Objective 1:*** *Determine if there is a statistically significant difference in runoff of alternative bacterial pathogen indicators, host-specific molecular biomarkers, and/or overtly pathogenic bacteria from manure-amended fields in the spring and winter.*

> ***Secondary Sub-objective 1a (non-critical measurement):*** *Determine if there is a statistically significant difference in the "edge of field" concentration of fecal bacteroides and/or host-specific molecular biomarkers in runoff following surface application of manure to frozen ground (winter application) versus thawed ground (spring application) Manure application and runoff sampling will coincide with that described in Primary Sub-objective 2a.*
>
> Approach: Collect runoff samples from the dustpan sample collectors following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring and test for fecal *Bacteroides* and host-specific fecal biomarkers using qPCR techniques. Manure application and runoff samples will coincide with those of Primary Sub-objective 2a. Inferential statistics will be used to test for differences in runoff of fecal *Bacteroides* and host-specific fecal biomarkers at the edge-of-field and are described in detail below.
>
> SO Hypothesis 1a-1: The "edge-of-field" concentration of fecal *Bacteroides* in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.
>
> SO Hypothesis 1a-2: The "edge-of-field" concentration of host-specific molecular biomarkers in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.
>
> SO Null Hypothesis 1a-1: The "edge-of-field" concentration of fecal *Bacteroides* in runoff does not differ between winter and spring manure applications.
>
> SO Null Hypothesis 1a-2: The "edge-of-field" concentration of host-specific molecular biomarkers in runoff does not differ between winter and spring manure applications.
>
> Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the "edge-of-field" fecal *Bacteroides* and/or host-specific fecal biomarkers in runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the

Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Fecal *Bacteroidetes* discharging from the manured fields are expected to range from 100 to 10,000,000 per 100 mL. The discharge of host-specific molecular biomarkers is unknown.

***Secondary Sub-objective 1b (non-critical measurement):*** *Determine if there is a statistically significant difference in the concentration of fecal bacteroides and/or host-specific molecular biomarkers in runoff from unmanured setbacks or VFSs following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). Manure application and runoff sampling will coincide with that described in Primary Sub-objective 2b.*

Approach: Collect runoff samples from the Coshocton wheel samplers at the down-slope end of the unmanured setbacks and VFSs and from dustpan sample collectors located at 11 m down-slope from the edge-of-field in the VFSs following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring (as defined in Primary Sub-objective 2a, above), and test for fecal *Bacteroides* and host-specific fecal biomarkers using qPCR techniques. Manure application and runoff samples will coincide with those of Primary Sub-objective 2b. Inferential statistics will be used to test for differences in fecal *Bacteroides* and host-specific fecal biomarkers in the unmanured setback or VFS runoff due to surface application to frozen or thawed ground, and are described in detail below.

SO Hypothesis 1b-1: The concentration of fecal *Bacteroides* in runoff collected down-slope of unmanured setbacks and VFSs following winter manure application is statistically significantly different from that resulting from spring manure application.

SO Hypothesis 1b-2: The concentration of host-specific fecal biomarkers in runoff collected down-slope of unmanured setbacks and VFSs following winter manure application is statistically significantly different from that resulting from spring manure application.

SO Null Hypothesis 1b-1: The concentration of fecal *Bacteroides* in runoff collected down-slope of unmanured setbacks and VFSs does not differ between winter and spring manure applications.

SO Null Hypothesis 1b-2: The concentration of host-specific fecal biomarkers in runoff collected down-slope of unmanured setbacks and VFSs does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the fecal *Bacteroides* and/or host-specific fecal biomarkers in unmanured setback and/or VFS runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Fecal *Bacteroidetes* discharging from the unmanured setback and VFS are expected to range from 100 to 10,000,000 per 100 mL. The discharge of host-specific molecular biomarkers is unknown.

***Secondary Sub-objective 1c (non-critical measurement):*** *Determine if there is a statistically significant difference in the "edge of field" concentration of Salmonella and/or E. coli O157:H7 in runoff following surface application of manure to frozen ground (winter application) versus thawed ground (spring application) Manure application and runoff sampling will coincide with that described in Primary Sub-objective 2a.*

Approach: Collect runoff samples from the dustpan sample collectors following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring and test for *Salmonella* and *E. coli* O157:H7 using cultivation and/or qPCR techniques. Manure application and runoff samples will coincide with those of Primary Sub-objective 2a. Inferential statistics will be used to test for differences in runoff of *Salmonella* and/or *E. coli* O157:H7 at the edge-of-field and are described in detail below.

SO Hypothesis 1c-1: The "edge-of-field" concentration of *Salmonella* in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

SO Hypothesis 1c-2: The "edge-of-field" concentration of *E. coli* O157:H7 in runoff resulting from winter manure application is statistically significantly different from that resulting from spring manure application.

SO Null Hypothesis 1c-1: The "edge-of-field" concentration of *Salmonella* in runoff does not differ between winter and spring manure applications.

SO Null Hypothesis 1c-2: The "edge-of-field" concentration of *E. coli* O157:H7 in runoff does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are

differences in the "edge-of-field" *Salmonella* and/or *E. coli* O157:H7 in runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Specific pathogen concentrations are expected to be less than 200 per mL to non-detect. It is unclear whether a significant difference in the runoff concentrations between manure applications can be observed, especially at these low concentrations.

***Secondary Sub-objective 1d (non-critical measurement):*** *in runoff from Determine if there is a statistically significant difference in the concentration of Salmonella and/or E. coli O157:H7 in runoff from unmanured setbacks or VFSs following surface application of manure to frozen ground (winter application) versus thawed ground (spring application). Manure application and runoff sampling will coincide with that described in Primary Sub-objective 2b.*

Approach: Collect runoff samples from the Coshocton wheel samplers at the down-slope end of the unmanured setbacks and VFSs and from dustpan sample collectors located at 11 m down-slope from the edge-of-field in the VFSs following surface application of manure to frozen ground in the winter and following an equivalent application to thawed ground in the spring (as defined in Primary Sub-objective 2a, above), and test for *Salmonella* and *E. coli* O157:H7 using cultivation and/or qPCR techniques. Manure application and runoff samples will coincide with those of Primary Sub-objective 2b. Inferential statistics will be used to test for differences in *Salmonella* and/or *E. coli* O157:H7 in the unmanured setback or VFS runoff due to surface application to frozen or thawed ground, and are described in detail below..

SO Hypothesis 1d-1: The concentration of *Salmonella* in runoff collected down-slope of unmanured setbacks and VFSs following winter manure application is statistically significantly different than that resulting from spring manure application.

SO Hypothesis 1d-2: The concentration of *E. coli* O157:H7 in runoff collected down-slope of unmanured setbacks and VFSs following winter manure application is statistically significantly different than that resulting from spring manure application.

SO Null Hypothesis 1d-1: The concentration of *Salmonella* in the unmanured setback and VFS runoff does not differ between winter and spring manure applications.

SO Null Hypothesis 1d-2: The concentration of *E. coli* O157:H7 in the unmanured setback and VFS runoff does not differ between winter and spring manure applications.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.). If a normal Q-Q plot suggests that the data are normally distributed, a 2-tailed *t*-test will be used to determine if there are differences in the *Salmonella* and/or *E. coli* O157:H7 in unmanured setback and/or VFS runoff resulting from winter and spring manure applications. If a normal Q-Q plot suggests that the data do not follow a normal distribution, the Wilcoxon test will be used as an alternative to the t-test. An $\alpha$ of 0.05 will be used to test for significant differences.

*Expected measurement range:* Specific pathogen concentrations are expected to be less than 200 per mL to non-detect. It is unclear whether a significant difference in the runoff concentrations between manure applications can be observed, especially at these low concentrations.

***Secondary Objective 2 (non-critical):*** *Determine the best-fit overall first-order COD degradation rate coefficient "k" associated with changes in the measured COD between the edge-of-field and discharge from 30 m unmanured setbacks or VFSs following surface application of manure in the winter and spring. Record changes in the soil physical properties that may be related to changes in the observed depletion of COD and therefore useful for the construction of more mechanistic models of COD runoff.*

***Secondary Sub-objective 1a (non-critical measurement):*** *Monitor the soil temperature and measure differences in the infiltration rates of thawed and frozen soil in the unmanured setbacks and VFSs.*

Approach: Monitor soil temperature during the period of study using soil temperature probes. A measure of infiltration will be the Soil and Water Conservation Service (SCS) curve number from the plots and watersheds where there is sufficient data collected from the sites under natural rainfall, and will be determined using the asymptotic method described by Hawkins (1993). Frost depth at the time of application of manure will be made by coring the frozen ground and determining soil water content at the time of application. Long-term soil temperature data is available from the NAEW archive and will be used to place newly collected soil temperature data in a historical context. If an insufficient number of rainfalls occur to compute curve numbers of the various watersheds, a sprinkling infiltrometer or double ring infiltrometer may be used to acquire data regarding the infiltration rate. However, it is unknown whether or not the equipment required for this measurement can be pushed into frozen ground.

SO Hypothesis 2: This is a data-driven objective rather than hypothesis-driven.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.).

*Expected measurement range:* Infiltration rates in the spring are expected to range from 0.5 to 7.5 cm per hour in the silt loams of the VFSs. In the winter months, the infiltration rate is expected to drop by approximately an order of magnitude.

***Sub-objective 2c (critical measurement):*** *Determine the best-fit first-order COD degradation rate coefficient "k" describing depletion of COD across the unmanured setbacks and VFSs following manure application to frozen ground (winter application) and thawed ground (spring application).*

Approach: Using the measured "edge of field" COD and unmanured setback and VFS runoff COD from sub-objectives 2a and 2b, determine the best fit "k" values for both winter and spring manure runoff using equation 4 of Appendix L.

PO Hypothesis 1b: This is a data-driven objective rather than hypothesis-driven.

Statistical Methods: Descriptive statistics will be used for reporting purposes (mean, standard deviation, range, etc.).

*Expected measurement range:* We expect the best fit "k" values for winter and spring to be near the value of 0.03 $min^{-1}$ as reported in Appendix L of Managing Manure Nutrients at Concentrated Animal Feeding Operations (EPA-821-B-04-006).

## *3.2 Sampling Strategy*

Various animal manures and process wastewaters will be sampled for large CAFOs to address primary objective 1a and to spread onto land to address the remaining objectives. Ideally, triplicates from different large CAFO sources of each animal manure or process wastewater type will be sampled, and the large CAFOs will be geographically distant (i.e. of different states in USEPA Region 5). However, the collection of the various samples will depend on the cooperation of agricultural producers, and cannot be controlled. Samples taken should be representative of various field-applied manures and process wastewaters and will include records on the farm source and location (which will be kept confidential), animal manure or process wastewater type, a brief description of the feeding practices (feed type, whether confined, grazed, free-range, etc.), and a short description of the treatment system employed (including process description, holding/storage time, hydraulic and/or sludge retention time if appropriate, composting time/temperature if appropriate, etc.). The manures should be tested prior to application to determine their percent solids, nitrogen, and phosphorus contents.

The sample collection points in the study unmanured setback and VFSs and the experimental watersheds at the NAEW are sited to intercept water flow at critical points in the system. Figures 1 and 2 show sampling locations that may be used in the study. One or more remote points in the waterways upstream or downstream of runoff stations 172 and 196 may also be considered at a later date for non-critical (molecular microbiological) measurements, such as runoff stations 172, 196, and/or several locations directly in the streams.

Samples will only be collected in the dustpan sample collectors and Coshocton Wheel samplers during runoff (discharge) events. It is presumed that the sample frequency will satisfy statistical concerns of the approaches outlined in Sections 3.1a – 3.1c. During runoff events, composite samples taken by Coshocton Wheel samplers at the unmanured setback and VFS runoff stations may be supplemented with samples of the "initial water flush" (initial runoff during the rising limb of the hydrograph), which would be taken by ISCO automated sampling equipment if budget permits. Water collected from the dustpan sample collectors at each cross-section of an unmanured setback or VFS will be composited prior to analysis. The fraction of water retained by the Coshocton wheel samplers or dustpan sample collectors will be agitated well prior to removing a representative sample for analysis.

Manually-collected samples that may be obtained for future studies at runoff stations 172 and 196 will be taken directly from the weir overflow. Any additional stream samples that we may be interested in acquiring upstream or downstream runoff stations 172 and 196 will be collected at 5-30 centimeters in depth using sterile techniques by opening the sterile collection bottle with gloved hands, submersing upside down to the appropriate sampling depth, up-righting the bottle and allowing it to fill. A small volume of water will be poured off the top of each sample bottle prior to capping (to approximately the demarcation line, see Section 4.4) to allow for headspace such that the sample can be agitated prior to analysis in the lab. If the depth of water is less than 5 centimeters at the point of sampling, the sample will be collected and the depth of water noted. Samples will not be collected from stream locations or from the runoff stations when the stream becomes stagnant or there is no flow over the weir. At present, we include no specific plans for grab sampling, other than for exploratory studies.

### *3.3 Monitoring Locations*

See Figures 1-7 and Sections 3.1 and 3.2

### *3.4 Sample Frequency*

Various animal manures and process wastewaters will be sampled once from each large CAFO source. The frequency of sampling will depend largely on the cooperation of livestock producers.

During runoff (discharge) events (we expect 4-5 total for each manure application), samples will be collected by the dustpan sample collectors, Coshocton Wheel Samplers,

and if budget permits ISCO automatic samplers, at each of the unmanured setback and VFS runoff locations. For these events, the dustpan sample collectors will each collect 10% and 1% of the intercepted flow, and the collected volume will be composited with that of the other dustpan collectors in the same transect on a volume-weighted basis prior to removing a single composited sample for each unmanured setback and VFS representative of the flow event. The Coshocton Wheel Samplers will automatically collect a flow-weighted composite sample representative of the runoff at each location and a single sample will be collected from each for each flow event. If used, the ISCO automatic samplers will be activated once a critical stage is reached at each autosampler station. Upon activation, the autosamplers will collect water at timed intervals (between 5 and 240 minutes) until the water stage begins to fall. We would expect three or more samples during the rising limb of the hydrograph at each runoff event. Therefore, each runoff event will at a minimum result in a single sample from each runoff sampling location (Coshocton Wheel Sampler or composited dustpan sample collectors). If ISCO automatic samplers are used, a minimum of four samples at each sample location will be generated for each rainfall event. Additional QC samples for microbiological analysis will include a field blank (each event) a matrix spike and laboratory replicates (two events per year), and a replicate field sample analyzed without the addition of reagents to identify potential interference from the sample matrix (two events per year). Sample holding time limitations restrict the possibility of holding reserve samples.

### *3.5 Measurements*

The following measurements will be recorded for this work:

**Critical Physical Measurements to be Obtained from the USDA-ARS staff:** Manure application field management details (crop cover, tillage, manure application rate, surface application method, manure type), vegetation on the VFSs (vegetation type, percent cover, height), precipitation intensity variation during runoff events, runoff volume from the experimental (cropped) watersheds and VFS plots during each runoff event, total volume collected in each dustpan sample collector during each runoff event, air temperature during events (recorded by the weather station), soil temperature, sample collection date and time for each sample, sample holding time and temperature (for COD analysis), SCS curve number for each watershed in the winter and spring, SCS curve number for each watershed used in the study calculated for till and no till conditions based on historical data.

**Critical Physical Measurements:** Sample holding time and shipping water blank temperature for samples for microbiological analysis.

**Critical Analytical Measurements to be obtained from the USDA-ARS staff:** COD (mg/L) of runoff samples, *E. coli* MPN per 100 mL (ColiSure), enterococci MPN per 100 mL (EnteroLert)

**Critical Analytical Measurements:** COD of manure and process wastewater samples, *E. coli* MPN per 100 mL (ColiSure), enterococci MPN per 100 mL (EnteroLert)

**Non-Critical Analytical Measurements:** fecal *Bacteroidetes* (qPCR), *E. coli* O157:H7 (PCR and/or qPCR), *Salmonella* (PCR and/or qPCR), antimicrobic resistance (broth microdilution), virulence factors of *E. coli* and enterococci (PCR-gel electrophoresis), host-specific molecular biomarkers (PCR and/or qPCR)

### *3.6 Evaluating Project Objectives*

See Section 3.1

## SECTION 4.0, SAMPLING PROCEDURES

### *4.1 Steady State Conditions*

The NAEW has been in operation for several years. There may be natural migration of wild animals through the study system over the period of investigation. However, the system in general is representative of agricultural and forested watersheds of the region.

The VFS study system is a new installation, and may not reach steady-state operation prior to manure application. However, untreated (no manure application) VFSs installed nearby to the study system will be used to control for the background condition. Vegetative cover will be in place (minimum of 80%) prior to initiation of the study.

Many samples collected will be grab samples taken from applied animal manures, process wastewaters, and running streams. As such, they will be assumed to be in a state of local steady flux over the time course of each sampling event. Overland flow samples collected by the Coshocton Wheel samplers and dustpan sample collectors will be composite samples, and will therefore represent the flow-weighted average of individual events. These systems (both natural and waste treatment systems) are inherently variable over the larger time frames of days to years, as they are sensitive to random events such as process failures, rainfall, and drought. Similarly, frozen soil conditions are subject to weather variations. These uncontrollable factors will remain limitations of the study.

### *4.2 Site Specific Factors*

None

### *4.3 Site Preparation*

The study VFSs at the NAEW are to be installed by the USDA-ARS support staff. Prior to initiation of the study, the VFSs must contain a minimum of 80% vegetative cover and have all sampling equipment installed. Maintenance of the VFSs (seeding, mowing, etc.) is at the discretion of the USDA-ARS. Coshocton Wheel Samplers, and if budget permits ISCO automatic samplers, at the VFS runoff and stream runoff sampling sites are to be installed by USDA-ARS support staff. If installed, the ISCOs will need to be calibrated to determine appropriate critical stages for initiation of sample collection. This will be performed at the discretion of USDA-ARS staff, with the goal of collecting three discrete one-liter samples on the rising limb of the hydrograph. The critical stages may need to be adjusted on a trial and error basis depending on data collected throughout the course of the study.

Manures and process wastewaters will be applied to the experimental (cropped) watersheds and VFS application areas at a rate described in Section 1.3 and Table 1. Manures will be analyzed before manure application to estimate annual nitrogen requirements for corn, then after application to verify the application rate used. Application method will be at the discretion of USDA-ARS staff, except that all manures

will be surface-applied (not incorporated). The USDA-ARS staff will not apply manure or process wastewater when there is a rain forecasted within 24 hours of application or in periods of active snow melt.

### *4.4 Sampling Procedures*

Manure and process wastewater samples will be collected from various large CAFOs pending access granted by the livestock operators. Ideally, three samples from different CAFOs for each animal manure or process wastewater type listed in primary objective 1a will be sampled. However, the collection of the various animal manures from different farms will depend on the cooperation of agricultural producers, and cannot be controlled. Manure and process wastewater samples will be taken from each facility as outlined in sections 3.2, 4.3, 4.8, and 4.9, and will be of material applied to land as a fertilizer. Any liquid manure or process wastewater obtained from storage will be fully agitated prior to sample collection and prior to filling manure handling equipment. Briefly, a total of 1000 mL (500 mL for microbiological analysis and 500 mL for COD analysis) of each liquid manure or process wastewater type will be collected in a sterile polypropylene bottle using aseptic techniques. The sample bottle will be pre-marked with lines indicating the correct fill level. For solid manures, approximately 500g will be collected and sealed in sterile polypropylene wide-mouth bottles using aseptic techniques. Solid manures should be taken from mixed piles ready for land application as opposed to individual animals. The leak-proof polypropylene bottles will be sealed in a zip lock bag, and shipped on ice in an insulated cooler overnight express to the USEPA, at which time the temperature will be recorded, and should be between >0ºC and 10ºC. Leaking or frozen samples will be discarded and the microbiological analysis will be prioritized for the remaining samples and/or for low-volume samples (<1000 mL) as shown in Table 2.

Water samples will be taken at each sampling location as outlined in sections 3.2, 4.3, 4.8, and 4.9, and otherwise will be collected in accordance with **EPA LT2 Draft methods** (attached). Briefly, 1000 mL samples (for microbiological analysis) and 500 mL samples (for COD analysis) at each location will be collected in sterile polypropylene bottles using aseptic techniques. The sample bottles will be pre-marked with lines indicating the correct fill level. All samples will be sealed and maintained between >0ºC - 10ºC (without freezing) until arrival in the laboratory, at which time the temperature of the temperature blank will be measured. Leaking or frozen samples will be discarded and the microbiological analysis will be prioritized for the remaining samples and/or for low-volume samples (<1000 mL) as shown in Table 2. The NAEW staff will control access to the various samplers and the weather station. The NOAA weather station data is available online at http:www.ncdc.noaa.gov/crn/hourly. Due to the uncontrollable nature of rainfall, some sample holding times may extend beyond 24 hours, but samples will not be analyzed later than 48 hours following collection.

Manual collection of stream samples: Potential manual samples will be taken at the runoff sampling stations in the receiving stream by allowing the sampling bottle to fill directly from the weir overflow. Water samples that may be taken from various points in the receiving stream will be taken by submerging the bottle to the appropriate depth (5-30

cm), allowing the bottle to fill, and pouring off the excess water to the demarcation line. Sample holding times should not exceed 24 hours prior to the onset of analysis.

Unmanured setback and VFS Dustpan Collectors: For each unmanured setback and VFS, the runoff water collected in the dustpan sample collectors of each transect will be composited in a sterile container of sufficient size to contain all collected runoff and mixed well prior to sampling. Two representative samples will be collected in sterile polypropylene bottles, 500 mL for COD analysis to be performed by the USDA-ARS, and 1000 mL to be shipped on ice priority overnight to the USEPA facility for microbiological analysis. The holding times in the dustpan collectors should be minimized as much as reasonably possible as they are not refrigerated during warmer weather. The time of sample collection and the time of removing a composite sample from the dustpan collectors will be recorded for each sample.

Coshocton Wheel Samplers: For each Coshocton Wheel sampler on the VFSs or experimental (cropped) watersheds, the retained water fraction will be mixed well prior to removing two representative samples in sterile polypropylene bottles, 500 mL for COD analysis to be performed by the USDA-ARS and 1000 mL to be shipped priority overnight on ice to the USEPA facility for microbiological analysis. The holding times in the Coshocton Wheel collection vessels should be minimized as much as reasonably possible as they do not have cooling capabilities. The time of sample collection and the time of removing a composite sample will be recorded for each sample.

ISCO Automatic samplers: If ISCO automatic samplers are used for runoff events, they will be set with sterile one liter bottles for sample collection prior to rainfall. Sample collection will be initiated once the stage at each station reaches a critical value (see section 4.3), after which discrete samples will be collected representing the rising hydrograph. The bottles will be exchanged with freshly prepared sterile bottles upon periodic collection of water samples, and the collected 1000 mL samples shipped on ice to the USEPA for microbiological analysis. The holding times in the autosamplers should be minimized as much as reasonably possible as they do not have cooling capabilities. The time of sample collection and the time of removal from the ISCO autosampler will be recorded for each sample. "Training" the ISCOs to collect samples on the appropriate intervals to capture the rising hydrograph will be a trial and error process. As such, the details of the sample collection scheme for the ISCOs will be at the discretion of the USDA-ARS NAEW staff.

**Table 2. Analysis priority for low-volume samples.**

| Priority | Volume | Analysis | Measure |
|---|---|---|---|
| 1 | 150 mL | *E. coli* plus dilutions | Critical |
| 2 | 300 mL | Enterococci plus dilutions | Critical |
| 3 | 550 mL | *E. coli* O157:H7 | Non-critical |
| 4 | 800 mL | *Salmonella* | Non-critical |
| 5 | 1000 mL | Fecal *Bacteroidetes* and source-specific molecular biomarkers | Non-critical |

### *4.5 Split Samples*

Not Applicable - Split samples will not be used.

### *4.6 Equipment Calibration*

The calibration and maintenance procedures for the equipment to be used in this study are shown in Table 3. The automatic samplers, weather station, incubators, dissolved oxygen meters, and IDEXX Quanti-Tray sealer and reader are used for the critical measurements of COD, *E. coli*, enterococci, VFS runoff volume, rainfall intensity, and temperature. The calibration and maintenance of the weather station and automatic samplers are at the discretion of the USDA-ARS NAEW and NOAA staff. In the event that an automatic sampling station fails to meet acceptable criteria at any sampling event, the stage will be recorded as unavailable. In the event that the weather station fails to meet acceptance criteria and/ or fails to collect critical data, the nearest National Weather Service Station data will be used and noted in the log books. In the event that the Idexx Quanti-Tray Sealer fails to perform, an alternative sealer located in house will be used to run the analyses. Several laboratory incubators are available in the event that one incubator fails to meet acceptance criteria.

All laboratory equipment will be maintained on a regular schedule. Laboratory equipment will be cleaned and inspected weekly for correct operation. Calibration and maintenance of the pH meter will be performed as noted in Table 3 and recorded in a log book. Incubator temperatures will be recorded once daily for each work day while in use to ensure that they maintain ± 0.5 ºC of the temperature required by the protocol. Refrigerator and freezer temperatures will also be recorded once daily for every work day in use. Acceptable temperature ranges for refrigerators will be between 1 – 5 ºC. For standard freezers, acceptable ranges will be between -20ºC ± 5 ºC. Ultra-low freezers will have an acceptable range of -70 ºC ± 10 ºC. "Frost-free" freezers will not be used as temperature fluctuations may lead to reagent degradation. Balances will be checked for calibration on a daily basis and recorded in a log book. Micropipettes will be calibrated on a yearly schedule.

### *4.7 Cross-Contamination*

The automatic samplers are installed permanently at each sampling location. No cross-contamination of samples due to moving sampling equipment is expected.

**Table 3. Equipment calibration and maintenance procedures**

| Measurement | Required Equipment | Calibration Procedures | Maintenance Procedures |
|---|---|---|---|
| ***Sampling Equipment:*** | | | |
| Rainfall, temperature | Weather Station | Manufac. [a.] | Manufac. |
| Stream and VFS overflow water samples | ISCO Automatic Sampler [b.] | Manufac. | Manufac. |
| | Coshocton Wheel Sampler | Manufac. | Manufac. |
| ***General Laboratory Equipment:*** | | | |
| pH | pH meter | Twice daily in appropriate buffers [c.] | Manufac. |
| Dissolved Oxygen | D.O. Meter | Daily | Manufac. |
| Centrifugation | Centrifuges | N/A | Manufac. |
| Weighing | Balances | Daily with appropriate weights | Manufac. |
| Culturing bacteria; Storage of Isolates; Media Preparation | Refrigerators/Incubators | USEPA, 2004 [d.] | Manufac. |
| | Hot Water Bath | N/A | Manufac. |
| | Shaker Table | N/A | Manufac. |
| | Micropipettes | USEPA, 2004 | |
| ***Specialized Laboratory Equipment:*** | | | |
| *E. coli*, and Enterococci | IDEXX Quanti-Tray sealer and reader | N/A | Manufac. |
| Antibiotic Resistance Analysis | Beckman Biomek 2000 Laboratory Automation Workstation | Manufac. | Manufac. |
| | Trek Diagnostics Nephelometer | Manufac. | Manufac. |
| Nucleic Acid Amplification and Detection | GeneAmp 9600 Thermalcycler | Manufac. | Manufac. |
| | Fisher Wide Format Gel Apparatus | N/A | Manufac. |
| | Cambrex FlashGel System | N/A | Manufac. |
| | ABI Prism 7000 Sequence Detection System | Manufac. | Manufac. |
| | Cepheid Smart Cycler II | Manufac. | Manufac. |
| | Spectrophotometer | USEPA, 2004 | Manufac. |

a. Manufac. = Calibration and maintenance to be performed as per manufacturers instructions.
b. As budget allows
c. The pH meter will be calibrated a twice daily and when calibration checks indicate probe drift. The buffers used for calibration will be dependent on the pH range of interest (pH 4 and 7 buffers for samples of pH $\leq$7; pH 7 and 10 buffers for samples of pH>7). The laboratory temperature will be entered into the meter prior to calibration if the meter does not have a temperature probe. Small quantities of buffer will be added to 10 mL disposable cups for calibrations and the probe rinsed with deionized water before insertion into each cup. Successful calibration will have a slope ranging from 95% to 105%. Probe calibration will be checked every twenty samples.
d. Calibration to be performed as outlined in Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples, USEPA, 2004, except that micropipettes will be calibrated once per year by a certified technician.

### *4.8 Representative Samples*

There is no way to guarantee the sampling is representative of normal conditions. However, the following sample collection rules will be used to minimize this fact:

*Collection rules to increase the likelihood of obtaining representative grab samples:*
Manure and process wastewater samples will be taken prior to field application. We aim to sample at least three sources for each waste type; however our access to different farm sources may be limited.

On occasion, additional stream samples may be taken for source tracking purposes. In these events, we will use runoff stations with weirs to the extent possible. However, direct stream samples may be taken. In these events, we will attempt to collect stream samples at a depth of 5-30 cm without disturbing the bottom sediments as described in Section 3.2. Infrequently, the stream may dry up at various sampling points and runoff stations such that a sample cannot be collected. In these events, samples will not be collected and will be noted in the sampling log book.

Samples will be removed from ISCO autosamplers during runoff events (if used) such that they can be analyzed as soon as possible following sample collection (ideally less than 24 hours, but no more than 48 hours). Samples collected in the dustpan collectors and Coshocton Wheel samplers will similarly be taken as soon as possible at the end of a runoff event for analysis. For runoff events that span more than a single day, daily composite samples will be taken and the system purged of its collected runoff to prepare for the following day's runoff. Physical sampling limitations during runoff events, such as samples initiated overnight, or lengthy rainfall or runoff events may result in sample holding times that exceed 24 hours considering sample shipping requirements. Multiple visits to each runoff sampling station may be required over a single event. A National Oceanic and Atmospheric Administration (NOAA) weather station is located at the NAEW next to the VFS plots (see Figure 1) and will record the rainfall amounts over each rainfall event. The data from this weather station can be accessed online at (http://www.ncdc.noaa.gov/crn/hourly). If budget permits ISCO autosamplers, our intent is to collect from selected watersheds and rainfall events a minimum of one sample representing total composited runoff (Coshocton Wheel sampler or dustpan collectors) and three samples during the rising hydrograph (one at the onset of rainfall and 2 additional samples as discharge increases and then peaks) from the runoff of each VFS and experimental (cropped) watershed.

*Inherent limitations to collecting representative samples:*
The NAEW is an established research facility on which several projects are being investigated simultaneously. Although the USDA-ARS staff will manage the VFS plots and experimental (cropped) watersheds as requested for our study, several other experimental watersheds and grazing areas are present in the NAEW that we do not have control over. Potential animal grazing, manure and other fertilizer applications, crop rotations, management of buffers, etc. are set by the site operators. Our work can only examine the potential movement of COD and microorganisms in the buffer system and watersheds of interest. We will also have no control over rainfall, drought, and other

weather factors in the various locations with concomitant changes in stream flow, overland flow, occurrence of no frozen soil or timing of frozen soil, and possible flooding/drying up of different sections of the watershed(s). We cannot control wildlife inputs in the system, and therefore the variation in these inputs will be considered inherent to this type of manure management system. Since we have no control over environmental conditions and many of the experimental watersheds outside our study but within the NAEW, there is no way to establish system replication on a larger watershed scale. This may lead to inherent variability in our measurements.

Many of the samples taken will be grab samples limited to one coordinate within a cross section of the stream, or in the case of the dustpan collectors – VFS or unmanured setback, in which they are taken. Discrete grab samples are inherently limited in describing "average" or "normal" conditions. The dustpan sample collectors are designed to collect 10% and 1% of all intercepted flow. There may be instances in which runoff is intense enough to overflow the collection units. These events will be noted when they occur, and will remain a limitation of the study. Further, we cannot control ISCO autosamplers (if used) to correspond to discharge measurements once triggered to collect samples. Because of this limitation, rainfalls of similar amounts but different intensities may yield a different number of total samples taken at different points on the hydrograph. We may (at a later date) attempt to explore fecal bacterial concentrations during discharge events downstream of the unmanured setbacks and VFSs in comparison to a co-located "background" watershed to better understand the influence the VFS runoff may be having on water quality in the receiving stream. Considering the limitations described above, there is no way to guarantee that individual sampling events will be representative of "normal" conditions.

### *4.9 Sample Quantities*

During runoff events, samples will be collected using the dustpan overland flow sample collectors, Coshocton Wheel Samplers, and if budget permits, ISCO automatic samplers. The dustpan sample collectors will collect 10% and 1% of all intercepted flow and will yield a single 1000 mL sample for microbiological analysis and 500 mL sample for COD analysis per sampling location and event. The Coshocton Wheel samplers will receive all overland flow discharging from the VFS, but will collect only a flow-weighted composite for analysis. A single 1000 mL sample for microbiological analysis and 500 mL sample for COD analysis per sampling location and event is the target volume. Once the critical stage is reached, the ISCO autosamplers will initiate periodic collection of 1000 mL samples as described in Section 4.4, and continue until the stage returns to the critical threshold. The total number of samples taken at each autosampler per runoff event can be as high as 24 samples per day, but we intend to retain a total of only 3 samples at each location representative of the rising hydrograph. The Coshocton Wheel Samplers and Dustpan Sample Collectors will be emptied daily or following each runoff event depending on the duration of the flow event as described in section 4.8. The ISCO autosamplers (if used) will be emptied periodically throughout the discharge event such that the analyses can be run within the recommended 24 hour holding time. Because of these limitations (also described in section 4.4), some samples may be analyzed past the

recommended 24 hour holding time. At a later date, we may also be interested in acquiring 1000 mL water samples from various locations downstream of the study systems for microbiological analysis as described in sections 3.2, 4.3, 4.4, and 4.8. Table 4 shows the sample quantities required for each analysis and the quantities to be collected, including QC samples for runoff sampling events. The sample volumes shown are based on estimates of the minimum concentrations of analytes we will observe, and thus may decrease contingent on actual concentrations measured following the first sampling event.

**Table 4. Runoff event-specific and QC sample quantities required.**

| Analyte | Required volume, mL (including dilutions) | # Replicates | Total Volume, mL (including dilutions) |
|---|---|---|---|
| ***Optional Downstream Samples:*** | | | |
| *E. coli* | 150 | 1 | 150 |
| Enterococci | 150 | 1 | 150 |
| Fecal *Bacteroidetes* and Source-specific molecular biomarkers | 200 | 1 | 200 |
| *E. coli* O157:H7 | 250 | 1 | 250 |
| *Salmonella* | 250 | 1 | 250 |
| | | **Total per location** | **1000** |
| | | **x 4 Locations** | **4000** |
| ***QC Samples (each collection date):*** | | | |
| Field Blank (all 6 analytes) | 1000 | 1 | 1000 |
| ***Biannual QC Samples:*** | | | |
| Matrix Spikes: | | | |
| *E. coli* (ColiSure) | 150 | 3 | 450 |
| Enterococci (EnteroLert) | 150 | 3 | 450 |
| Fecal *Bacteroidetes* and Source-specific molecular biomarkers | 200 | 3 | 600 |
| *E. coli* O157:H7 | 250 | 3 | 750 |
| *Salmonella* | 250 | 3 | 750 |
| Laboratory Replicates: | | | |
| *E. coli* (ColiSure) | 150 | 3 | 450 |
| Enterococci (EnteroLert) | 150 | 3 | 450 |
| Replicate field sample (no reagents) | 100 | 1 | 100 |
| | | **Total per location** | **4000** |
| | | **x 4 Locations (2 VFS and 2 stream)** | **16000** |
| ***Runoff Event Samples:*** | | | |
| COD / $BOD_u$ | 500 | 1 | 500 |
| *E. coli* (ColiSure) | 150 | 1 | 150 |
| Enterococci (EnteroLert) | 150 | 1 | 150 |
| Fecal *Bacteroidetes* and Source-specific molecular biomarkers | 200 | 1 | 200 |
| *E. coli* O157:H7 | 250 | 1 | 250 |
| *Salmonella* | 250 | 1 | 250 |
| | | **Total per location** | **1500** |
| | | **x6 VFSs x3 locations/VFS** | **27000** |
| | | **+ 4x experimental (cropped) watersheds x2 locations each** | **39000** |
| | | **+ 6x VFS ISCOs x3 per event (optional)** | **66000** |

### *4.10 Sample Containers*

All environmental water samples will be collected in sterile 500 mL or 1000 mL polypropylene bottles as described in section 4.4, capped, sealed in a leak-proof (zip-lock) bag, placed on ice in an insulated cooler and either taken to the NAEW laboratory for COD analysis or shipped priority overnight to the USEPA AWBERC Facility as described in section 4.14 for microbiological analysis (or COD analysis on manure and process wastewater samples).

### *4.11 Sample Identification*

Samples of manures and process wastewaters taken from various animal facilities will be accompanied with details of the waste type, storage time, and treatment processes. Sample ID codes for these facilities will be of the format:

**LRPCD-"Facility ID"-"animal source"-"date of sample collection in MMDDYY format"-"time of sample collection (military)"-"sample bottle number"/"total sample bottles"**

For example, the sample ID for a dairy cattle manure sample collected from CAFO XYZ where a lagoon is used on June 20, 2005 at 4:45 pm would be:

LRPCD-CAFOXYZ-dairy-062005-1645-1/1

The IDs for the different animal sources are:

| | |
|---|---|
| Manure from mature dairy cows: | dairy |
| Swine manure: | swine |
| Manure from egg-laying chickens: | layers |
| Turkey manure: | turkey |
| Manure from beef cattle: | beef |
| Manure from broiler chickens: | broilers |
| Egg wash process wastewater: | eggpww |
| Cattle open lot process wastewater: | cattlepww |
| Process wastewater from feed storage: | feedpww |

The specific sample ID codes for each environmental water sample taken at the NAEW will be in the format:

**LRPCD-NAEW-"sample location"-"date of sample collection in MMDDYY format"-"time of sample collection (military)"-"sample bottle number"/"total sample bottles"**

For example, the sample ID for a sample collected from the overflow of VFS1 at the NAEW on June 20, 2005 at 4:45 pm would be:

LRPCD-NAEW-VFS1 overflow-062005-1645-1/1

The specific ID codes for each environmental isolate (*E. coli* or *Enterococcus* spp.) originating from the NAEW will refer to the date the sample was taken, and will be in the format:

**LRPCD-NAEW-"sample location"-"date of sample collection in MMDDYY format"- EC(for *E. coli*) or ENTERO (for *Enterococcus* spp.)-"isolate number"**

For example, the ID codes for the 9th isolate of *E. coli* and the 6th isolate of *Enterococcus* spp. from samples collected at NAEW-VFS4EOF (edge of field) on June 20, 2005 at 4:15 pm would be:

LRPCD-NAEW-VFS4EOF-062005-EC-9
LRPCD-NAEW-VFS4EOF-062005-ENTERO-6.

### *4.12 Sample Preservation*

All environmental samples will be sealed in a leak-proof container, placed in a zip lock bag, placed immediately on ice in an insulated and covered cooler, and maintained at >0 ºC – 10 ºC for a period less than 24 hours prior to analysis in the laboratory. On some runoff events, samples may be analyzed past the recommended holding time, ***but no later than 48 hours from sample collection***. Environmental isolates of *E. coli* will be stored for short periods (<1 month) on nutrient agar slants in a refrigerator at 4ºC until antimicrobic resistance analysis and PCR detection of virulence traits. Environmental isolates of *Enterococcus* spp. will be stored for short periods (<1 month) on brain-heart infusion agar slants in a refrigerator at 4ºC until antimicrobic resistance analysis and PCR detection of virulence traits. Long term storage of environmental isolates is described in section 4.16.

### *4.13 Holding Time Requirements*

Analyses of environmental samples for *E. coli* and enterococci should begin no later than 24 hours after sample collection as indicated in the **EPA LT2 Draft methods** (attached). On specific runoff events, it may be necessary to analyze some samples past the 24 hour holding time requirement. Based on parallel studies comparing 24 and 48 hour holding times, we have noted only a slight decline in *E. coli* populations (less than 10%) and very slight increase in enterococci populations (less than 5%) when samples are properly refrigerated. This is within the sample variability expected in the methods. We have not studied the effect of holding time on COD or $BOD_u$, but this will remain a limitation of this study. Samples should be analyzed for COD/$BOD_u$ no later than 24 hours following collection.

### *4.14 Packing and Shipping*

Each sample container will be labeled with the sample location and identification, collection date and time, and sampler's initials using a waterproof marker prior to sample collection. Clear packing tape should be used to cover labels and prevent them from

getting wet and falling off. All sample information will also be documented in field log books to serve as a sample inventory, including identification of control samples. Samples will be tracked by site, sample location, and collection date and time.

Samples will be capped tightly, placed into a zip lock bag, and immediately placed on ice in an insulated cooler and maintained at >0 ºC – 10 ºC until receipt in the laboratory. A temperature blank (water) in the same volume container as the samples will be placed into the cooler with the samples such that the temperature can be measured upon receipt in the lab. All ice should be placed in large zip lock bags and the ice and samples placed into garbage bag tied at the top to prevent leakage into the sample cooler. If the samples are to be overnight shipped, the coolers should be taped to prevent them from opening during shipment. The shipment method should be selected so that the samples arrive at the AWBERC facility within 24 hours of collection (overnight priority).

### *4.15 Chain of Custody*

A chain of custody form will be initiated upon collection of the samples. The chain of custody form will include the sample information described in section 4.14 as well as the location of the samples within the shipping containers (i.e. which samples are in which coolers) and the name and date of all personnel who accepted possession of the samples between collection and delivery to the laboratory. Any specific information regarding sample integrity and/or changes in the status of the samples (labeling, seals, records, spillage, etc.) will also be recorded. At the laboratory, the chain of custody will be used to ensure all samples collected were delivered and to prioritize the order of analysis.

### *4.16 Sample Archives*

Environmental samples will not be archived due to holding time restriction prior to analysis. Environmental isolates of *E. coli* and *Enterococcus* spp. will be stored frozen in glycerol at -70ºC for long-term archival purposes.

### *4.17 Field Records*

Field records will include the name and contact information of the recorder, identification of the sample site, names of participants in the site sampling event, dates, weather conditions, and any other pertinent environmental information. Specific details about the operation of the facility will be recorded including any important changes in the operation/condition of the BMPs. All samples will be recorded as described in sections 4.11 and 4.15.

Field records will be maintained by each individual on-site. Following sampling events, copies of the field notes of each individual will be distributed to all pertinent parties along with an executive summary of the information. Individual field notes will be used to summarize and cross validate field records. A binder containing copies of all field records will be constructed and maintained.

## SECTION 5.0, TESTING AND MEASUREMENT PROTOCOLS

### *5.1 Methods*

Upon receipt in the USEPA AWBERC laboratory, the water temperature blank will be removed from each cooler and the temperature measured by inserting a thermometer. If the temperature is within the acceptable range of >1ºC – 10 ºC, the samples will be analyzed immediately or placed in a refrigerator at 4 ºC for a short time until analyses can be initiated (24-48 hours from sample collection). If the temperature of the blank is above the acceptable range, the samples will be analyzed, but all data generated from the samples will be flagged. Samples that are frozen will be discarded.

Prior to analysis, sample bottles will be shaken by hand a minimum of 100 times (approximately one to two minutes) to homogenize the samples. Appropriate aliquots (or serial dilutions) will be taken from each sample bottle to be used for multiple analyses. For low volume samples (<1000 mL), the analysis priority for microbiological characterization is listed in Table 2. Samples will be shaken 20-25 times between each aliquot removed, and will not be left above 10ºC longer than 30 minutes at a time.

Table 5 summarizes the critical and non-critical analytical measurement methods to be used in the study. ***Critical analytical measurements*** include total and nonsettleable COD and $BOD_u$, *E. coli*, and Enterococci. Total and nonsettleable COD and $BOD_u$ will be measured according to Standard Methods for the Examination of Water and Wastewater and the methods of Tchobanoglous and Schroder (1985). *E. coli* will be measured using the ColiSure® (Quanti-Tray®) chromogenic substrate MPN technique (SOP attached). Enterococci will be measured using the EnteroLert® (Quanti-Tray®) chromogenic substrate MPN technique (SOP Attached). The ColiSure and Quanti-Tray technologies have been approved for use by the USEPA for measuring total coliforms and *E. coli* under the Total Coliform Rule (40 CFR141.21(f)(3) and (f)(6)(iv)). The EnteroLert® and Quanti-Tray® technologies have been approved by the USEPA for measuring Enterococci in ambient waters under the Guidelines Establishing Test Procedures for the Analysis of Pollutants; Analytical Methods for Biological Pollutants in Ambient Water; Final Rule (40 CFR Part 136 Vol. 68, No. 139). ***Non-critical analytical measurements*** may include quantitative detection of specific pathogens using molecular microbiological methods, antimicrobial resistance monitoring, microbial source tracking with host-specific biomarkers, and the detection of virulence genes in *E. coli* and *Enterococcus* environmental isolates.

*Non-critical methods description:*
Fecal *Bacteroidetes*, *E. coli* O157:H7, and *Salmonella* spp. will be measured qualitatively and/or quantitatively in the "edge of field" runoff, VFS runoff, and stream waters using PCR and/or real-time qPCR methods as shown in Table 5. Real time qPCR amplifications will be run on an ABI Prism 7000 sequence detection system (Applied Biosystems) or Cepheid SmartCycler II. The PCR mixture, thermal cycling parameters, and fluorogenic probe and primer sets to be used are described by others (summarized in Table 6). Standard curves for the fecal pathogens will be constructed with known quantities of the template DNA in PCR water, and will be compared to those spiked into the environmental matrix. Standard curves for the fecal Bacteroidetes will also be

constructed in PCR water, and the results compared to those of a matrix spike. Control strains and no template controls will be used in each 96 well plate for quality control purposes.

Although the authors shown in Table 5 describe the sensitivity of their methods, we will perform several small studies to establish our own detection limits because PCR sensitivity is affected by DNA extraction, which in turn is affected by specific factors unique to the environmental matrix. In some instances, low concentrations of specific pathogens such as *E. coli* O157:H7 anticipated in the water samples may require pre-enrichment in selective media and/or nested PCR to obtain a low enough sensitivity for detection (Chern et al., 2002). Pre-enrichment of the fecal *Bacteroidetes* is not anticipated, as these organisms constitute a large fraction of total fecal bacteria (Dick and Field, 2004).

PCR biomarker analysis will be used at the site to link changes in fecal bacterial indicators observed across the VFSs and in the receiving stream waters to the field applied manure fertilizers. Several previously published biomarkers will be investigated targeting human and livestock animal hosts. Table 7 summarizes the methods. For the 16S rDNA-targeted biomarkers, concentration of bacteria by membrane filtration followed by direct DNA extraction and PCR may be appropriate as copies of these genes are present in every cell (Bernhard and Field, 2000a,b). However, for methods targeting virulence genes, not every cell will harbor the gene of interest (GOI). Therefore, nested PCR and/or pre-enrichment in mTEC media (*E. coli*) or mE media (*Enterococcus*) may be required to obtain the appropriate sensitivity (Chern et al., 2002). As with the real-time PCR methods, we will perform several small studies to establish our own detection limits respective to our environmental matrices. Molecular ladders and control strains will be incorporated routinely into gels for QA/QC purposes. Gel patterns will be digitally recorded for archival purposes.

**Table 5. Measurements and methods to be used in the study**

| Measurement | Method |
|---|---|
| ***Critical*** | |
| *E. coli* | ColiSure® and Quanti-Tray® (SOP Attached) |
| Enterococci | EnteroLert® and Quanti-Tray® (SOP Attached) |
| Total COD | Standard Methods for the Examination of Water and Wastewater |
| Nonsettleable COD | Tchobanoglous and Schroder (1985) |
| ***Non-Critical*** | |
| Fecal *Bacteroidetes* | qPCR, Dick and Field (2004) |
| *E. coli* O157:H7 | qPCR, Ibekwe et al. (2002), Chern et al. (2004) |
| Salmonella | qPCR, Fukushima et al (2003) |
| *E. coli* Antimicrobial Resistance | Broth Microdilution, SensiTitre® Veterinary Gram Negative NARMS Panels (Trek Diagnostic Systems Method Attached) |
| *Enterococcus* spp. Antimicrobial Resistance | Broth Microdilution, SensiTitre® Veterinary Gram Positive NARMS Panels (Trek Diagnostic Systems Method Attached) |
| *E. coli* Virulence Factors | PCR/Gel Electrophoresis, López-Saucedo et al. (2003), Obi et al. (2004) |
| *Enterococcus* spp. Virulence Factors | PCR/Gel Electrophoresis, Vankerckhoven et al. (2004), Bittencourt de Marques et al (2004), Dupré et al (2003), Eaton and Gasson (2001) |
| Human Biomarker | PCR/Gel Electrophoresis, Scott et al. (2005), Bernhard and Field (2000a,b) |
| Cattle Biomarker | PCR/Gel Electrophoresis, Khatib et al. (2002), Bernhard and Field (2000a,b) |
| Swine Biomarker | PCR/Gel Electrophoresis, Khatib et al. (2003) |

**Table 6. Real-Time qPCR: intended (potential) gene targets, oligonucleotide probes and primers, and reaction conditions**

| Target/ Locus | Oligonucleotide Sequence (5'→3') | | PCR Conditions* | | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | $C_{pr}$† (µM) | $T_a$‡ (°C) | $Mg^{2+}$ (mM) | Amplicon (bp) | |
| **Fecal Indicators** | | | | | | | |
| Fecal *Bacteroidetes* 16S rDNA | f:<br>r:<br>probe: | GCT-CAG-GAT-GAA-CGC-TAG-CT<br>CCG-TCA-TCC-TTC-ACG-CTA-CT<br>CAA-TAT-TCC-TCA-CTG-CTG-CCT-CCC-GTA | 0.4<br><br>0.2 | 60 | 3.5 | NR§ | Dick and Field (2004) |
| ***E. coli* spp.** | | | | | | | |
| EHEC Shiga-like toxin 1 | stx1-f:<br>stx1-r:<br>probe: | GAC-TGC-AAA-GAC-GTA-TGT-AGA-TTC-G<br>ATC-TAT-CCC-TCT-GAC-ATC-AAC-TGC<br>TGA-ATG-TCA-TTC-GCT-CTG-CAA-TAG-GTA-CTC | 0.3<br><br>0.1 | 55 | 5 | 150 | Ibekwe et al. (2002) |
| EHEC Shiga-like toxin 2 | stx2-f:<br>stx2-r:<br>probe: | ATT-AAC-CAC-ACC-CCA-CCG<br>GTC-ATG-GAA-ACC-GTT-GTC-AC<br>CAG-TTA-TTT-TGC-TGT-GGA-TAT-ACG-AGG-GCT-TG | 0.3<br><br>0.1 | 55 | 5 | 200 | Ibekwe et al. (2002) |
| *E. coli* O157:H7 Attachment and effacement | eae-f:<br>eae-r:<br>probe: | GTA-AGT-TAC-ACT-ATA-AAA-GCA-CCG-TCG<br>TCT-GTG-TGG-ATG-GTA-ATA-AAT-TTT-TG<br>AAA-TGG-ACA-TAG-CAT-CAG-CAT-AAT-AGG-CTT-GCT | 0.3<br><br>0.1 | 55 | 5 | 106 | Ibekwe et al. (2002) |
| ***Salmonella* spp.** | | | | | | | |
| *Salmonella* spp. Invasion-associated locus | invA-f:<br>invA-r:<br>SYBR | GTG-AAA-TTA-TCG-CCA-CGT-TCG-GGC-AA<br>TCA-TCG-CAC-CGT-CAA-AGG-AAC-C | 1 | 55 | 2 | 284 | Fukushima et al. (2003) |

* Anticipated PCR conditions based on referenced methods
† $C_{pr}$ = Primer concentration in multiplex PCR reaction
‡ $T_a$ = Primer annealing temperature
§ NR = Not reported

**Table 7. Host-Specific Biomarkers: intended (potential) gene targets, oligonucleotide primers, and reaction conditions**

| Target Locus | Oligonucleotide Sequence (5'→3') | | PCR Conditions* | | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | $C_{pr}$† (µM) | $T_a$‡ (°C) | $Mg^{2+}$ (mM) | Amplicon (bp) | |
| ***Human*** | | | | | | | |
| *Bacteroidetes* 16S rDNA | HF183-f:<br>BAC708-r: | ATC-ATG-AGT-TCA-CAT-GTC-CG<br>CAA-TCG-GAG-TTC-TTC-GTG | 10 | 59 | 1.5 | 525 | Bernhard and Field (2000a,b) |
| *Enterococcus faecium* esp | EfESP-f<br>EfESP-r | TAT-GAA-AGC-AAC-AGC-ACA-AGT<br>ACG-TCG-AAA-GTT-CGA-TTT | 0.3 | 58 | 1.5 | 680 | Scott et al. (2005) |
| ***Cattle*** | | | | | | | |
| *Bacteroidetes* 16S rDNA | CF128-f:<br>BAC708-r: | CCA-ACY-TTC-CCG-WTA-CTC<br>CAA-TCG-GAG-TTC-TTC-GTG | 10 | 58 | 1.5 | 580 | Bernhard and Field (2000a,b) |
| *Bacteroidetes* 16S rDNA | CF193-f:<br>BAC708-r: | TAT-GAA-AGC-TCC-GGC-C<br>CAA-TCG-GAG-TTC-TTC-GTG | 10 | 55 | 1.5 | 515 | Bernhard and Field (2000a,b) |
| *E. coli* LTIIa | LTIIa-f:<br>LTIIa-r: | GGG-TGT-GCA-TTT-CAG-CGA-C<br>TGG-TAT-ATT-CCG-GGT-GGA-CG | NR§ | 61 | 2.0 | 358 | Khatib et al (2002) |
| ***Swine*** | | | | | | | |
| *E. coli* STII | STII-f:<br>STII-r: | TGC-CTA-TGC-ATC-TAC-ACA-AT<br>TAG-AGA-TGG-TAC-TGC-TGG-AAG | NR | 47 | 2.0 | 113 | Khatib et al. (2003) |

* Anticipated PCR conditions based on referenced methods
† $C_{pr}$ = Primer concentration in multiplex PCR reaction
‡ $T_a$ = Primer annealing temperature
§ NR = Not reported

We may select at random *E. coli* and *Enterococcus* isolates from positive Quanti-Tray wells at various points in the systems for downstream microbial analyses. *E. coli*-positive wells from ColiSure® Quanti-Trays® will be randomly sampled via sterile syringe inserted through the well and screened for *E. coli*-positive colonies on CHROMagar ECC. Although most pathogenic *E. coli* strains can be screened in this manner, we are aware that some pathogenic *E. coli* serotypes lack β-glucuronidase and therefore do not cleave MUG (they result in coliform positive, but *E. coli* negative wells in the ColiSure® assay). This will remain a limitation of the study.

Enterococci-positive wells from EnteroLert® Quanti-Trays® will be randomly sampled via sterile syringe inserted through the well and screened for *Enterococcus*-positive colonies on CHROMagar Orientation. One *E. coli* or *Enterococcus* colony per plate will be selected and grown to logarithmic phase (~8 hours) at 35ºC in 20 mL Luria Broth (LB) (*E. coli*) or brain-heart infusion (BHI) broth (*Enterococcus*) on a shaker table. The cells will be harvested and washed 3X in 1x PBS and a loop stored on LB agar slants (*E. coli*) or BHI agar slants (*Enterococcus*) for up to a month at a time. Frozen glycerol stocks of each isolate will also be prepared from log-phase cultures for archival purposes.

*E. coli* and *Enterococcus* isolate may be screened for resistance to antimicrobials of clinical importance using the broth microdilution method (Trek Diagnostic Systems, attached) on 96-well SensiTitre® Veterinary Gram-Negative NARMS panels (*E. coli*) or SensiTitre® Veterinary Gram-Positive NARMS panels (*Enterococcus*). The plates will be read manually as described in the method, where a visible "button" on the bottom of a well indicates growth. Table 8 lists the antimicrobials and concentrations on the NARMS panels and breakpoints of each antimicrobial relevant to *E. coli* or Enterococcus.

The selected colonies may also be screened for virulence factors that may be indicative of specific *E. coli* or *Enterococcus* pathotypes using PCR and gel-electrophoresis techniques listed in Table 5 and summarized in Table 9. Prior to DNA extraction, a portion of logarithmic-phase *E. coli* or *Enterococcus* culture will be transferred to a fresh tube, pelleted, and resuspended in guanidine isothiocyanate buffer. DNA will be extracted either by using the boiling technique or, if poor quality DNA inhibits the PCR reaction, using an appropriate commercial nucleic acid extraction kit (such as the DNeasy tissue kit (QIAGEN, Valencia, CA)) as per manufacturer's instructions. All of the primers will be verified on control strains independently, and we will attempt to run the reactions in multiplex, as suggested by the authors of these methods. We will investigate the suggested primer and magnesium concentrations to determine if they are optimal for multiplexing in our laboratory prior to running the analyses based on the equivalency of band sizes given equal amounts of template DNA and judged by quantitative molecular ladders. As with the biomarker analyses, molecular ladders and control strains will be incorporated routinely into gels for QA/QC purposes and gel patterns will be digitally recorded for archival purposes.

**Table 8. Antimicrobials on the NARMS panels and NCCLS breakpoints**

| Antimicrobial | Concentration (µg/mL) | Breakpoints† (µg/mL) | | |
|---|---|---|---|---|
| | | Susceptible | Intermediate | Resistant |
| ***Gram Negative NARMS, Broth Microdilution (E. coli)*** | | | | |
| Amikacin | 0.5 – 32 | ≤ 16 | 32 | ≥ 64 |
| Ampicillin | 1 – 32 | ≤ 8 | 16 | ≥ 32 |
| Amoxicillin/Clavulanic Acid | 1/0.5 – 32/16 | ≤ 8/4 | 16/8 | ≥ 32/16 |
| Ceftriaxone | 0.5 – 64 | ≤ 8 | 16 – 32 | ≥ 64 |
| Chloramphenicol | 2 – 32 | ≤ 8 | 16 | ≥ 32 |
| Ciprofloxacin | 0.015 – 4 | ≤ 1 | 2 | ≥ 4 |
| Trimethoprim/sulfamethoxazole | 0.12/2.38 – 4/76 | ≤ 2/38 | | ≥ 4/76 |
| Cefoxitin | 0.5 – 32 | ≤ 8 | 16 | ≥ 32 |
| Gentamicin | 0.25 – 16 | ≤ 4 | 8 | ≥ 16 |
| Kanamycin | 8 – 64 | ≤ 16 | 32 | ≥ 64 |
| Nalidixic Acid | 0.5 – 32 | ≤ 16 | | ≥ 32 |
| Sulfisoxazole [a.] | 16 – 512 | ≤ 256 | | ≥ 512 |
| Streptomycin | 32 – 64 | ≤ 32 | | ≥ 64 |
| Tetracycline | 4 – 32 | ≤ 4 | 8 | ≥ 16 |
| Ceftiofur [b.] | 0.12 – 8 | ≤ 2 | 4 | ≥ 8 |
| ***Gram Positive NARMS, Broth Microdilution (Enterococcus spp.)*** | | | | |
| Bacitracin[c.] | 8 – 128 | ≤ 32 | 64 | ≥ 128 |
| Chloramphenicol | 2 – 32 | ≤ 8 | 16 | ≥ 32 |
| Erythromycin | 1 – 8 | ≤ 0.5 | 1 – 4 | ≥ 8 |
| Flavomycin [c.] | 1 – 32 | ≤ 8 | 16 | ≥ 32 |
| Penicillin | 0.5 – 16 | ≤ 8 | | ≥ 16 |
| Daptomycin [d.] | 0.5 – 16 | ≤ 8 | | ≥ 16 |
| Quinipristin/dalfopristin | 1 – 32 | ≤ 1 | 2 | ≥ 4 |
| Tetracycline | 4 – 32 | ≤ 4 | 8 | ≥ 16 |
| Vancomycin | 0.5 – 32 | ≤ 4 | 8 – 16 | ≥ 32 |
| Lincomycin [c.] | 1 – 32 | ≤ 8 | 16 | ≥ 32 |
| Tylosin tartrate [c.] | 0.25 – 32 | ≤ 8 | 16 | ≥ 32 |
| Ciprofloxacin | 0.12 – 4 | ≤ 1 | 2 | ≥ 4 |
| Linezolid | 0.5 – 8 | ≤ 2 | 4 | ≥ 8 |
| Nitrofurantoin | 2 – 64 | ≤ 32 | 64 | ≥ 128 |
| Kanamycin [c.] | 128 – 1024 | ≤ 128 | 256 | ≥ 512 |
| Gentamicin | 128 – 1024 | < 500 | | ≥ 500 |
| Streptomycin [c.] | 512 – 2048 | < 1000 | | ≥ 1000 |

† NCCLS M100-S14

a. Based on NCCLS breakpoints for sulfamethoxazole

b. NARMS, 2002 annual report

c. NARMS, Retail Meat 2002

d. Alder, J., T. Li, D. Yu, L. Morton, J. Silverman, X. Zhang, I. Critchley, and G. Thorne (2003) Analysis of Daptomycin Efficacy and Breakpoint Standards in a Murine Model of *Enterococcus faecalis* and *Enterococcus faecium* Renal Infection, *Antimicrobial Agents and Chemotherapy*, **47**(11): 3561-3566.

**Table 9.** ***E. coli*** **virulence traits: intended (potential) gene targets, oligonucleotide primers, and reaction conditions**

| Target/Locus | Oligonucleotide Sequence (5'→3') | | PCR Conditions* | | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | $C_{pr}$† (µM) | $T_a$‡ (°C) | $Mg^{2+}$ (mM) | Amplicon (bp) | |
| ***Reaction 1 (Multiplex)*** | | | | | | | |
| Heat-labile toxin | LT-f:<br>LT-r: | GGC-GAC-AGA-TTA-TAC-CGT-GC<br>CGG-TCT-CTA-TAT-TCC-CTG-TT | 0.2 | 50 | 1.5 | 450 | López-Saucedo et al. (2003) |
| Heat-stable toxin | STaP-f:<br>STaP-r: | ATT-TTT-CTT-TCT-GTA-TTG-TCT-T<br>CAC-CCG-GTA-CAA-GCA-GGA-TT | 0.26 | 50 | 1.5 | 190 | López-Saucedo et al. (2003) |
| Bundle-forming pilus | Bfp-f:<br>Bfp-r: | AAT-GGT-GCT-TGC-GCT-TGC-TGC<br>GCC-GCT-TTA-TCC-AAC-CTG-GTA | 0.1 | 50 | 1.5 | 324 | López-Saucedo et al. (2003) |
| Attachment and effacement | eaeA-f:<br>eaeA-r: | GAC-CCG-GCA-CAA-GCA-TAA-GC<br>CCA-CCT-GCA-GCA-ACA-AGA-GG | 0.155 | 50 | 1.5 | 384 | López-Saucedo et al. (2003) |
| Shiga-like toxin 1 | stx1-f:<br>stx1-r: | CTG-GAT-TTA-ATG-TCG-CAT-AGT-G<br>AGA-ACG-CCC-ACT-GAG-ATC-ATC | 0.155 | 50 | 1.5 | 150 | López-Saucedo et al. (2003) |
| Shiga-like toxin 2 | stx2-f:<br>stx2-r: | GGC-ACT-GTC-TGA-AAC-TGC-TCC<br>TCG-CCA-GTT-ATC-TGA-CAT-TCT-G | 0.1 | 50 | 1.5 | 255 | López-Saucedo et al. (2003) |
| Invasion-associated locus | ial-f:<br>ial-r: | GGT-ATG-ATG-ATG-ATG-AGT-CCA<br>GGA-GGC-CAA-CAA-TTA-TTT-CC | 0.41 | 50 | 1.5 | 650 | López-Saucedo et al. (2003) |
| ***Reaction 2*** | | | | | | | |
| Enteroaggregative gene | eaeC-f:<br>eaeC-r: | CTG-GCG-AAA-GAC-TGA-ATC-AT<br>CAA-TGT-ATA-GAA-ATC-CGC-TGT-T | 0.2 | 53 | 2.0 | 630 | Obi et al. (2004) |
| ***Reaction 3 (Multiplex)*** | | | | | | | |
| Cytotoxic necrotizing factor 1 | cnf-f:<br>cnf2-r: | GCT-CAA-CGA-GAC-TAT-GCT-CTG<br>ACG-CTG-CTA-AGT-ACC-TCC-TGG | 0.2 | 63 | 2.0 | 278 | Obi et al. (2004) |
| Cytotoxic necrotizing factor 2 | cnf2-f:<br>cnf2-r: | GTG-AGG-CTC-AAC-GAG-ATT-ATG-CAC-TG<br>CCA-CGC-TTC-TTC-TTC-AGT-TGT-TCC-TC | 0.03 | 63 | 2.0 | 839 | Obi et al. (2004) |

* Anticipated PCR conditions based on referenced methods
† $C_{pr}$ = Primer concentration in multiplex PCR reaction
‡ $T_a$ = Primer annealing temperature

**Table 9. (cont.) *Enterococcus spp.* virulence traits: intended (potential) gene targets, oligonucleotide primers, and reaction conditions**

| Target/Locus | Oligonucleotide Sequence (5'→3') | | PCR Conditions* | | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | $C_{pr}$† (µM) | $T_a$‡ (°C) | $Mg^{2+}$ (mM) | Amplicon (bp) | |
| ***Reaction 1 (Multiplex)*** | | | | | | | |
| Enterococcal surface protein | esp-f:<br>esp-r: | AGA-TTT-CAT-CTT-TGA-TTC-TTG-G<br>AAT-TGA-TTC-TTT-AGC-ATC-TGG | 0.2 | 56 | 2.5 | 510 | Vankerckhoven et al. (2004) |
| Gelatinase | gelE-f:<br>gelE-r: | TAT-GAC-AAT-GCT-TTT-TGG-GAT<br>AGA-TGC-ACC-CGA-AAT-AAT-ATA | 0.1 | 56 | 2.5 | 213 | Vankerckhoven et al. (2004) |
| Cytolysin activator | cylA-f:<br>cylA-r: | ACT-CGG-GGA-TTG-ATA-GGC<br>GCT-GCT-AAA-GCT-GCG-CTT | 0.2 | 56 | 2.5 | 688 | Vankerckhoven et al. (2004) |
| Aggregation substance | as-f:<br>as-r: | GCA-CGC-TAT-TAC-GAA-CTA-TGA<br>TAA-GAA-AGA-ACA-TCA-CCA-CGA | 0.1 | 56 | 2.5 | 375 | Vankerckhoven et al. (2004) |
| Hyaluronidase | hyl-f:<br>hyl-r: | ACA-GAA-GAG-CTG-CAG-GAA-ATG<br>GAC-TGA-CGT-CCA-AGT-TTC-CAA | 0.1 | 56 | 2.5 | 276 | Vankerckhoven et al. (2004) |
| ***Reaction 2 (Multiplex)*** | | | | | | | |
| Cytolysin secretion/transporter | cylB-f:<br>cylB-r: | ATT-CCT-ACC-TAT-GTT-CTG-TTA<br>AAT-AAA-CTC-TTC-TTT-TCC-AAC | 0.4 | 55 | 1.5 | 843 | Bittencourt de Marques et al (2004) |
| Post-translational modification of cytolysin | cylM-f:<br>cylM-r: | CTG-ATG-GAA-AGA-AGA-TAG-TAT<br>TGA-GTT-GGT-CTG-ATT-ACA-TTT | 0.4 | 56 | 1.5 | 742 | Bittencourt de Marques et al (2004) |
| Enterolysin A | enlA-f:<br>enlA-r: | TTC-TTC-TTA-TTC-TGT-CAA-CGC-AGC<br>GAC-TGT-GAA-ATA-CCT-ATT-TGC-AAG-C | 0.4 | 59 | 1.5 | 960 | Bittencourt de Marques et al (2004) |
| Accessory colonization factor | ace-f:<br>ace-r: | AAA-GTA-GAA-TTA-GAT-CCA-CAC<br>TCT-ATC-ACA-TTC-GGT-TGC-G | 0.8 | 56 | 1.5 | 320 | Dupré et al (2003) |
| ***Reaction 3 (Multiplex)*** | | | | | | | |
| E. faecalis cell-wall adhesin | efaAfs-f:<br>efaAfs-r: | GAC-AGA-CCC-TCA-CGA-ATA<br>AGT-TCA-TCA-TGC-TGT-AGT-A | 0.4 | NR§ | 1.5 | 705 | Eaton and Gasson (2001) |
| E. faecium cell-wall adhesion | efaAfm-f:<br>efaAfm-r: | AAC-AGA-TCC-GCA-TGA-ATA<br>CAT-TTC-ATC-ATC-TGA-TAG-TA | 0.4 | NR | 1.5 | 735 | Eaton and Gasson (2001) |

* Anticipated PCR conditions based on referenced methods
† $C_{pr}$ = Primer concentration in multiplex PCR reaction
‡ $T_a$ = Primer annealing temperature
§ NR = Not reported

### *5.2 Unproven Methods*

All critical measurements are measured with established (proven) methods.

### *5.3 Calibration Procedures*

All sampling bottles, glassware, media, and disposables will be sterilized prior to use. Vials, media, nutrient solutions, and glassware will be sterilized in an autoclave for 20 minutes at 121ºC. Laboratory disposables such as syringes and filters, bottle filters, and microfuge tubes will be purchased in sterile packaging and RNase/DNase-free where appropriate.

All equipment will be maintained and calibrated as described in section 4.6 and Table 3. Equipment that cannot meet acceptance criteria for calibration will be checked for malfunctions and repaired. Affected samples will be re-analyzed where possible. Preferably, processing of samples will begin not more than 24 hours from collection. If problems are encountered in analysis, alternate equipment in-house will be used where possible to process samples before the 24 hours of sample collection. Otherwise, environmental samples will be held at 4 ºC for up to 48 hours and processed. Samples will not be processed after 48 hours of collection.

## SECTION 6.0, QA/QC CHECKS

### *6.1 Acceptance Criteria for QA Objectives*

See Table 10 for acceptance criteria and corrective actions for critical COD, *E. coli*, and enterococci measurements. QA guidelines for the weather station, Coshocton wheel samplers, and ISCO automatic samplers will be based on the manufacturer's performance guidelines and are at the discretion of the USDA-ARS NAEW and NOAA staff who will maintain the equipment. In the event that an automated sampling station fails to meet acceptable criteria or is unavailable for sampling at any sampling event, the sample will be recorded as unavailable. In the event that the weather station fails to meet acceptance criteria and/or fails to collect critical data, the nearest National Weather Service Station data (Coshocton, Ohio) will be used and noted in the log books. In the event that the dissolved oxygen meter/probe or the Idexx Quanti-Tray Sealer fails to perform, an alternative meter/probe or sealer located in house will be used to run the analyses. Several laboratory incubators are available in the event that one incubator fails to meet acceptance criteria.

See **SensiTitre 18-24 hour MIC susceptibility plates** for acceptance criteria and corrective actions for non-critical, antimicrobial resistance measurements (SOP attached). Quality assurance for nucleic acid techniques (equipment, reagents, workflow, QA/QC procedures, corrective actions, etc.) will follow **EPA Office of Groundwater and Drinking Water and Office of Research and Development guidelines** (EPA, 2004; attached). Quality control procedures will include negative control wells in every PCR reaction to test for nucleic acids contamination. If contamination is detected, reagents will be discarded and prepared fresh. Where commercial kits/reagents are used, manufacturer QA/QC protocols will be followed.

Molecular ladders and control strains will be routinely incorporated into the PCR and gel detection procedures for QA purposes. Control strains will be routinely incorporated into real-time PCR reactions for QA procedures. Where positive control strains fail detection or negative control wells display detection in gels or real-time reactions, the results will be rejected, all reagents and potentially contaminated equipment will be investigated, the problem corrected, and the analyses will be reran where possible.

Real-time PCR reactions will be run with a passive reference dye (ROX) incorporated into the master mix to compensate for small well-to-well fluorescence variations and other variations that may interfere with quantification when using the ABI Sequence Detection System 7000. The Cepheid SmartCycler II does not have this capability. For detecting pathogens, MGB probes will be used to reduce background fluorescence and improve detection of low copy number targets. Template DNA for the standard curves will be measured spectrophotometrically to ascertain the copy numbers per stock volume then serially diluted to prepare standards of known quantities. Standard curves will be run on every real-time PCR plate for the ABI Sequence Detection System 7000 and weekly during analysis using the Cepheid SmartCycler II. Standard curves will be constructed in PCR-grade water and compared to a matrix spike, and will be rejected and

reran if the efficiency of the reaction is greater than 1 or less than 90% or if the $R^2$ is less than 0.9.

For general microbiological procedures related to testing antimicrobial resistance and nucleic acid techniques, all preparations of selective media will be checked with positive and negative controls. Sterility of agar plates will be checked by incubating a plate from each batch for 24 hours at 35 ºC. Batches that do not pass quality control checks will be discarded. Sterility of dilution water used for microbiological analyses will be tested by filtering 50 mL of dilution water through a 0.2 µM membrane filter, placing the filter on a TSA plate, and incubating at 35 ºC for 24 hours. Contaminated dilution water will be discarded and prepared fresh. Sterility of membrane filters will be performed for each filter lot by placing a single filter on a TSA plate and incubating at 35 ºC for 24 hours. Contaminated lots will be discarded. A UV light box will be used to disinfect filtration units between samples. The sterility of these units will be tested during each filtration event by filtering a sterile blank and running the relevant analyses. Where contamination is present, affected data will be flagged.

### *6.2 Additional QA Objectives*

Most probable numbers of *E. coli* and enterococci by the ColiSure/Quanti-Tray and EnteroLert/Quanti-Tray methods are more statistically relevant when the number of wells yielding positive results are between 20- 80% of the total number of wells (97). Serial dilutions will be prepared in an attempt to yield plates that fall within this range. For replicates that yield low numbers in some and no growth in the other plates without dilution, an MPN of <1 per 100 mL will be recorded. All statistical analyses will be performed with $\alpha \leq 0.05$.

**Table 10a. Quality assurance objectives for critical measurements**

| QA Objective | Acceptance Criteria | Corrective Action |
|---|---|---|
| ***Field Samples*** | | |
| Sample identification | Sample ID, date/time of sample collection, and initials | Samples analyzed but marked as unknown samples |
| Sample integrity | Collection vessels not leaking | Discard leaking samples |
| Holding time | Holding time <48 hours | Discard if >48 hrs. |
| Temperature blank | 0 ºC < temperature blank < 10 ºC | Analyze samples >10ºC, but flag data. Discard frozen samples. |
| ***Reagents and consumables*** | | |
| **Sterility checks:** | | |
| New lots ColiSure/EnteroLert | No growth with sterile dilution water for each new lot. | Discard contaminated lots |
| New lots sterile sample bottles | No growth with sterile dilution water for each new lot. | Discard contaminated lots |
| New lots of Quanti-Trays | No growth with sterile dilution water for each new lot. | Discard contaminated lots |
| Dilution water | Run analyses with sterile dilution water each day analyses are performed. No growth, color change, or fluorescence. | Discard and prepare fresh dilution water. Adjust and flag affected data if appropriate. Discard affected data where contamination is severe. |
| **Autofluorescence/color checks:** | | |
| New lots of ColiSure and EnteroLert dehydrated media | Run one analysis with sterile dilution water for each new lot. No color change or fluorescence should be observed. | Discard contaminated lots |
| **ColiSure +/- Controls:** | | |
| *Escherichia coli* (from Quanti-Cult® QC Kit) | Analyze each lot with sterile dilution water + loop of control strain. Color change = (+), Fluorescence = (+). | Discard lots that do not yield a positive (+) reaction for both coliforms and *E. coli*. |
| *Kleibsella pneumoniae* (from Quanti-Cult® QC Kit) | Analyze each lot with sterile dilution water + loop of control strain. Color change = (+), Fluorescence = (-). | Discard lots that do not yield a positive (+) reaction for coliforms and negative reaction for *E. coli*. |
| Pseudomonas aeruginosa (from Quanti-Cult® QC Kit) | Analyze each lot with sterile dilution water + loop of control strain. Color change = (-), Fluorescence = (-). | Discard lots that do not yield a negative (-) reaction for both coliforms and *E. coli*. |
| **EnteroLert +/- Controls:** | | |
| *Enterococcus faecium* (ATCC# 35667) | Analyze each lot with sterile dilution water + loop of control strain. Fluorescence = (+). | Discard lots that do not yield a positive (+) reaction for enterococci. |
| *Serratia marcescens* (ATCC# 43862) | Analyze each lot with sterile dilution water + loop of control strain. Fluorescence = (-). | Discard lots that do not yield a negative (-) reaction for enterococci. |
| *Aerococcus viridans* (ATCC# 10400) | Analyze each lot with sterile dilution water + loop of control strain. Fluorescence = (-). | Discard lots that do not yield a negative (-) reaction for enterococci. |

**Table 10b. Quality assurance objectives for critical measurements**

| QA Objective | Acceptance Criteria | Corrective Action |
|---|---|---|
| ***Analytical Equipment*** | | |
| Refrigerators, daily record | 1 ºC < T < 5 ºC | Adjust temperature setting, repair if needed |
| Incubators, daily record | ± 0.5 ºC | Adjust temperature setting and tolerance, repair if needed |
| Thermometers, yearly record | Annual calibration at relevant temperature against NIST-traceable thermometer. Calibration = ± 1 ºC | Discard and replace thermometers that do not meet acceptance criteria |
| pH meter, daily record | Calibration slope between 95% to 105% | Recalibrate if possible, replace probe if cannot meet acceptance criteria |
| DO meter, daily record | As per manufacturer's instructions, minimum of 100% saturation | Recalibrate if possible, replace probe if cannot meet acceptance criteria |
| Autoclave | Autoclave thermometer verifies 121ºC was achieved | Re-sterilize in an alternative autoclave and repair malfunctioning unit |

### *6.3 QA Procedures*

Specific QA procedures not described in sections 6.1 or 6.2 include the following:

Environmental Samples: Temperature blanks will be analyzed upon receipt of the samples in the laboratory by removing the blank from the cooler and immediately immersing a thermometer into the sample and reading the temperature. The temperature of the sample blank will be recorded on the relevant sample data sheets.

Critical Measurements: QA procedures for ColiSure and EnteroLert methods are detailed in their respective SOPs (attached). QA procedures for COD and $BOD_u$ are detailed in Standard Methods for the Analysis of Water and Wastewater.

Thermometers: The thermometers will be calibrated once per year against an NIST standard reference thermometer at the relevant temperature by placing both thermometers into the refrigerator/incubator/water bath of interest and comparing the readings. If a thermometer varies from the NIST reference thermometer by more than ±1 ºC, it will be adjusted if possible (some digital thermometers) or discarded. The reference thermometer will be checked every five years and discarded if it is off by more than ±1 ºC.

Refrigerators, Incubators, and Water Baths: The temperature of refrigerators, incubators and water baths may be monitored by attached digital thermometers or immersed mercury thermometers, and should be recorded in their associated logbooks daily.

pH meter: Calibration of the pH meter should be performed twice daily when in use or more frequently when calibration checks indicate probe drift. The buffers used for calibration will be dependent on the pH range of interest (pH 4 and 7 buffers for samples of pH ≤7; pH 7 and 10 buffers for samples of pH>7). The laboratory temperature will be entered into the meter prior to calibration if the meter does not have a temperature probe. Small quantities of buffer will be added to 10 mL disposable cups for calibrations and the probe rinsed thoroughly with deionized water before insertion into each cup. Successful calibration will have a slope ranging from 95% to 105%. Probe calibration will be checked every twenty samples. Probes that cannot achieve a slope between 95 and 105% will be discarded and replaced.

Micropipettes: Calibration of micropipettes will be routinely performed as outlined in **EPA Office of Groundwater and Drinking Water and Office of Research and Development guidelines**, except that micropipettes will be calibrated only once per year by a certified technician (USEPA, 2004; attached).

PCR Analytical Equipment: Calibration of thermal cycling equipment and real-time PCR machines will be performed as per manufacturer's instructions. Servicing of the machines will be performed by certified technicians.

### *6.4 QC Checks and/or Procedures*

Field blanks will be taken at each sampling event to identify potential contamination problems with sampling, and should result in no detection of *E. coli*, enterococci, fecal *Bacteroidetes*, or specific pathogens. Where field blanks display contamination, the associated data from the sampling event will be flagged if the contamination is greater than 1% of the measured concentration. Twice per year, samples will be taken from each matrix (stream water, VFS influent ("edge of field"), and VFS effluent water), composited in the lab, and used to test for method precision of the ColiSure and EnteroLert techniques using laboratory triplicates. There should be no outliers as identified using a Grubbs test at $\alpha$=0.01. Further, matrix spikes for the these methods will be constructed by collecting a second set of samples from each matrix then spiking the replicates with known concentrations of *E. coli* and enterococci in the laboratory prior to analysis. The background (indigenous) populations will be subtracted from the matrix spike value and compared to samples run in parallel with sterile laboratory water spiked with the same concentrations. If the matrix does not affect sample results, a student's t-test should result in no difference at $\alpha$=0.01. If the matrix is found to affect sample results, the results will be adjusted accordingly and the frequency of matrix spikes adjusted to every sampling event. Matrix effects will also be investigated by analyzing replicate samples with the ColiSure and EnteroLert methods, but without the reagents to test for autofluorescence and/or color. Quanti-Trays displaying slight coloration or fluorescence may be used as comparators for reading the samples. Twice per year, all samples for COD will be run in triplicate on composite samples and the average value reported. There should be no outliers for each sample location as identified using a Grubbs test at $\alpha$=0.01. Laboratory blanks for COD will be run for each sampling event, and should result in a COD of less than 0.01 mg/L. The study design incorporates field duplicates and a background (no-application) control plot for VFS influent and effluent samples, although these replicates will be used to identify variability in the runoff from the VFSs, not for specific QC validation.

For non-critical real-time PCR measurements, matrix spikes will be used to identify and correct for matrix effects. All real-time PCR samples will be run in triplicate to determine method precision and identify potential extraction and pipetting errors in sample setup. Threshold concentrations ($C_T$) that exceed 0.5 $C_T$ from the other two will be discarded, and the remaining data points averaged to quantify copy numbers of the gene of interest (GOI). If all three replicates are more than 0.5 $C_T$ from each other, the analysis will be rerun. For presence/absence test (PCR-gel detections of virulence traits and antimicrobial resistance screening on *E. coli* and enterococci isolates), 5% of the isolates will be duplicated. Duplicates that do not agree will be analyzed a third time for a consensus.

### *6.5 QC Check and/or Procedure Frequencies, Acceptance Criteria, and Corrective Actions*

See Table 11.

**Table 11. QC checks for critical and non-critical measurements**

| Analyte | QC Check | Frequency | Acceptance Criteria | Corrective Action |
|---|---|---|---|---|
| ***Critical Measurements*** | | | | |
| *E. coli* and enterococci | Laboratory triplicates | 2 events per year, one for each matrix corresponding to matrix spikes | No outliers, Grubb's test $\alpha$=0.01 | Remove outlier and use the average of the remaining data |
| | Field blank | One at every sampling event | Target=No detect, acceptance=<1% of lowest sample value | If >1% of sample value, report, but flag, associated data |
| | Matrix Spike | 2 events per year, performed in triplicate for each matrix plus at each runoff event sampling | No difference from lab control, t-test, $\alpha$=0.01 | Adjust samples accordingly, increase frequency of collection of matrix spikes to each sampling event if necessary. |
| | Field replicate, no ColiSure or EnteroLert | 2 events per year | No color or fluorescence | Use as comparators for environmental samples. Increase frequency of collection of to each sampling event. |
| $COD/BOD_u$ | Laboratory Triplicates | 2 events per year | No outliers, Grubb's test $\alpha$=0.01 | Remove outlier and use the average of the remaining data |
| | Laboratory blank | One at every sampling event | $COD/BOD_u$<0.01mg/L | If >1% of sample value, report, but flag, associated data |
| ***Non-critical Measurements*** | | | | |
| Real-time PCR: Fecal *Bacteroides*, *E. coli* O157:H7, *Campylobacter*, and *Salmonella* | Field blank | One at every sampling event | No detect | If >1% of sample values, report, but flag, data |
| | Matrix Spike | 2 events per year | No difference from lab control, t-test, $\alpha$=0.01 | Adjust samples accordingly, increase frequency of collection of matrix spikes to each sampling event if necessary. |
| | Laboratory triplicates | Every sample | $\leq 0.5\ C_T$ | Remove outlier, average remaining data, re-run reaction if all three samples are $\geq 0.5\ C_T$ of each other |
| | No template controls | One in each real-time PCR reaction | No detection | Rerun analyses due to potential contamination problem |
| | Standard Curve | One in each real-time PCR reaction | $0.9 \leq E \leq 1$; $R^2 \geq 0.95$ | Identify and correct problem (software, outliers, pipetting errors, machine, DNA template purity, primers/probes, contamination, polymerase, etc.), rerun if necessary |
| PCR-Gel detection of virulence traits: *E. coli* and enterococci | Lab duplicates | 5% of strains tested, selected at random | Agreement between presence/absence of each GOI | Run a third time for a consensus |
| Antimicrobial Resistance: *E. coli* and Enterococci | Lab duplicates | 5% of strains tested, selected at random | Agreement between MIC and susceptible/resistant of each antimicrobic | Run a third time for a consensus, average the three data points for MIC if necessary |

## SECTION 7.0, DATA REPORTING, DATA REDUCTION, AND DATA VALIDATION

### *7.1 Reporting Requirements*

Table 12 summarizes the reporting requirements for critical and non-critical measurements. Briefly, COD will be reported as mg/L. Environmental populations of *E. coli* and enterococci will be reported as MPN/100 mL. Fecal *Bacteroidetes*, *E. coli* O157:H7, and *Salmonella* will be reported as copy numbers per 100 mL, and a corresponding approximation of copy numbers per cell will accompany the copy number values. *E. coli* and *Enterococcus* isolates screened for antimicrobial resistance will be reported as susceptible, intermediate, or resistant to each antimicrobial, and the corresponding MIC of each antimicrobial will be reported in µg/mL if appropriate (within the range of the microtitre dilution broth). *E. coli* and *Enterococcus* isolates screened for virulence genes will be reported as positive and negative for each GOI. Where statistical approaches are used, measures of significance will be reported.

### *7.2 Expected Deliverables from Each Organization*

The USDA-ARS NAEW staff will be responsible for all sample collection, initiation of chain of custody forms, preservation and shipment of samples, and maintaining field notebooks. The preserved samples will be delivered via overnight express to the following address:

Dr. Shane Rogers
US Environmental Protection Agency
National Risk Management Research Laboratory
26 W. Martin Luther King Dr., MS421
Cincinnati, OH 45268

All samples should arrive with a chain of custody in the laboratory within 20 hours of sample collection to allow for processing before the 24 hour deadline. During specific runoff events, it is understood that sample shipment may be delayed due to sample collection limitations. Under these circumstances, the samples should arrive in the laboratory no later than 44 hours after sample collection. Copies of all pertinent sample record logs/field notes should be sent to John Haines and Shane Rogers within one month of each sampling event. In the event that runoff occurs between Thursday evening and Sunday morning such that samples cannot be shipped to arrive in the AWBERC facility within 44 hours of sample collection, the USDA-ARS staff will measure *E. coli* and enterococci at the NAEW facility. The USDA-ARS NAEW staff will also be responsible for measurement of COD in all collected runoff samples. Weather data and VFS and stream discharge data will be acquired through the USDA-ARS NAEW staff.

John Haines and Shane Rogers will be responsible for providing training in proper techniques for sample collection and shipment, if necessary, as well as providing sample collection logs, chain of custody forms, sample collection bottles, and shipping materials. Drs. Haines and Rogers will also be responsible for receiving samples in the laboratory, performing all analyses except for COD on runoff samples, and maintaining laboratory and QA/QC notebooks.

**Table 12. Reporting requirements for critical and non-critical measures**

| Matrix | Measurement | Units |
|---|---|---|
| ***Critical Measurements*** | | |
| All | Temperature | Degrees Celsius (ºC) |
| Aqueous | Discharge (stream, ditch, or tile) | Cubic meters per day (cmd) |
| N/A | Precipitation | Centimeters (cm) |
| N/A | Sample holding time | Hours |
| Aqueous | *E. coli* | Most probable number per 100 milliliters (MPN / 100 mL) |
| Aqueous | Enterococci | MPN / 100 mL |
| Aqueous | COD, $BOD_u$ | Milligrams per liter (mg/L) |
| ***Non-critical Measurements*** | | |
| Aqueous | Fecal *Bacteroidetes* | Copy number / 100 mL |
| Aqueous | *E. coli* O157:H7 | Copy number / 100 mL |
| Aqueous | Salmonella | Copy number / 100 mL |
| Aqueous | Campylobacter | Copy number / 100 mL |
| N/A | Antimicrobial resistant *E. coli* and *Enterococcus spp.* | Susceptible (S), intermediate (I), or resistant (R), plus MIC in µg/mL where appropriate |
| N/A | Virulence genes in *E. coli* and *Enterococcus spp.* | Presence (+) / absence (-) |

## *7.3 Data Reduction*

All field conditions and sampling information will be recorded in field notebooks. All experimental conditions and results will be reported in laboratory notebooks. Replicates will be recorded individually, but reported as averages with error bars to represent data ranges. A computer database will be constructed using Microsoft Access to store data in a format that is easily retrievable. Computer spreadsheets will be used to reduce and analyze data (Microsoft Excel). Spreadsheets and the computer database will be analyzed for errors by the researcher recording the data, and all results checked against laboratory or field notebook values a minimum of twice for each experiment/sampling event. Descriptive statistics will be used to reduce data across sampling events for reporting purposes. Averages will be reported with error bars to represent data ranges (standard deviations, 95% confidence intervals, etc.).

## *7.4 Data Validation*

Spreadsheets and the computer database will be analyzed for errors by the researcher recording the data, and all results checked against laboratory or field notebook values a minimum of twice for each experiment/sampling event. Reported values in publications and technical presentations will be checked a minimum of twice against the database values to ensure accuracy in reporting. Field, laboratory, and QA/QC notebooks will be archived to be available as a reference for validating reported results.

## *7.5 Data Storage*

See sections 7.3 and 7.4

### *7.6 Product Documents*

It is expected that the results of this study will be presented by the primary investigators at one or more technical conferences and in at least one peer-reviewed journal article. The results will be used in part to address winter application guidelines for animal manure and to fill in data gaps in Appendix L in Managing Manure Nutrients at Concentrated Animal Feeding Operations (EPA-821-B-04-006). The results will also be used, at least in part, to address USEPA LRPCD APM 516 for the Water Quality MYP Long Term Goal 3: Demonstration of a strategy to evaluate BMP effectiveness in watersheds impaired by fecal contamination.

## SECTION 8.0, ASSESSMENTS

### *8.1 Scheduled Audits*

No audit is planned

### *8.2 Corrective Actions*

The primary investigators will review the QA notebooks and QC results to decide if the data is valid and to ensure proper corrective actions have been or are being performed when necessary.

### *8.3 Responsible Parties*

The primary investigators (John Haines and Shane Rogers) will be responsible for implementing corrective actions for sample processing and analysis. The primary investigators for the USDA-ARS (James Bonta, Martin Shapitalo, and Lloyd Owens) will be responsible for implementing corrective actions related to environmental sampling where appropriate (as outlined in above and or deemed necessary by Drs. Rogers and Haines).

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