III. MATERIALS AND METHODS

A. Research and Site Description

ARA was used to determine sources of fecal contamination in the watersheds of the Coan and Little Wicomico Rivers. Both rivers are located in Virginia's NorthernNeck, Northumberland County. Northumberland County was established in 1648, and is a peninsula that borders the Potomac and Rappahannock Rivers and the Chesapeake Bay. Northumberland County's 570 square kilometers (220 square miles) consists mainly of flat to gently rolling county side bounded by 705 kilometers (438 miles) of shoreline on inlets, creeks, the Potomac River and the Chesapeake Bay (Northumberland Chamber of Commerce, 2003). The Coan River empties into the lower Potomac River near Caliao and the Little Wicomico empties into the Chesapeake Bay at Smith Point, where the Potomac River and Chesapeake Bay combine. The survey area consists of 59.268 square kilometers (22.885 square miles) for the Coan River and 39.171 square kilometers (15.125 square miles) for the Little Wicomico River. The Division of Shellfish Sanitation established these survey boundaries. Both rivers are polluted with fecal matter, and contain shellfish beds that have been closed because of high levels of fecal coliforms.

The Virginia Department of Health with the Division of Shellfish Sanitation collected most of the fecal and water samples. The sampling was part of routine DSS monitoring that has occurred for the past 10-12 years and these stations have been used by DSS over the same period. Howard Kator, at the Virginia Institute of Marine Science (VIMS), also collected additional water samples as well as Dr. Hagedorn and Cheryl Szeles during trips to the watersheds. All fecal samples and water samples were delivered to Howard Kator's laboratory at VIMS within 6 hours of collection. The

samples were filtered and the number of *Escherichia coli* present was determined using modified m-TEC agar (BBL Inc.). Filter plates were then shipped in plastic Hazardous Material containers to Virginia Tech by overnight delivery. Nine sites were sampled in the Coan River Watershed (Figure 1), and six sites were sampled in the Little Wicomico River Watershed (Figure 2) over a 12-month period from September of 2001 to August of 2002.

B. Construction of the Known Source Library

1. Known source sample collection

Fecal samples from known sources in the watershed were collected to build the library of known source profiles. Isolates from nine different sources were collected in Northumberland County, VA, from September 2001 to August 2002 to build a known source library. Sources included cow (*Bos taurus*), horse (*Equus caballus*), dog (*Canus familiaris*), deer (*Odocoileus virginianus*), muskrat (*Ondatra zibethica*), raccoon (*Procyon lotor*), wild turkey (*Meleagris gallapavo*), sea gull (*Larus argentatus smithsonianus*), double crested cormorant (*Phalacrocorax auritus*), Canadian goose (*Anser domesticus*), domestic Canadian goose (*Branta canadenis*) and human (*Homo sapien*). These sources were split into 5 specific groups, Bird, Human, Livestock, Pets, and Wildlife. Livestock samples were collected from local farms, wildlife samples were from locations where wildlife have been observed and the scat samples could be identified, and human sources were collected from septic tank pump out trucks, and volunteers. Solid fecal samples were collected in sterile Whirl-Pac bags and liquid fecal samples were collected in sterile polystyrene bottles. All samples were placed on ice in coolers and transported to the laboratory. The DSS as well as Dr. Hagedorn and Cheryl

Szeles collected known source samples. Known source samples were diluted and plated on m-TEC agar (BBL Inc.) to obtain isolates. Details for collecting and making isolations from the known source fecal samples have been reported (Hagedorn *et al*., 1999).

2. Isolation of *Escherichia coli*

Colonies of *Escherichia coli* were removed from the m-TEC (BBL Inc.) plates with sterile toothpicks and transferred to 96-well microtiter plates containing a 0.2-milliliter solution of Coliert broth (IDEXX). The microtiter plates were then incubated at 37º C for 24 to 48 hr. After incubation most if not all of the wells turned yellow in color and the color change indicated lactose fermentation by coliforms. If no color change occurred, the isolate was not used in the analysis (although it was replica-plated). *Escherichia coli* can be separated from fecal coliforms by examining the plate in the dark under Ultra-Violet light. All wells containing isolates that are methlumbelliferyl-ß-D-glucuronide (MUG), positive (Difco Laboratories) will yield a blue green fluorescence. Cultures that are methlumbelliferyl-ß-D-glucuronide (MUG) negative were checked on EMB agar (Sigma) to determine if they are truly MUG (-) *Escherichia coli*.

3. Antibiotic Resistance Analysis

Twenty-eight concentrations of 7 antibiotics were used to determine antibiotic resistance patterns in fecal *Escherichia coli*. Antibiotic stock solutions were prepared from commercial products (Sigma, Inc.) as indicated in Table 3. Each of the twenty-eight antibiotic/ concentrations was added separately to flasks of autoclaved and cooled Trypticase Soy Agar (TSA) from the stock solutions to achieve desired concentration, Table 4, and then poured into sterile 15 x 100 millimeter petri dishes. Control plates (no

antibiotics) were included with each set. Isolates were transferred from the micro-well plate using a stainless steel 48-prong replica plater (Sigma Inc.). The replicator is flame sterilized (95% ethanol) after inoculation of each TSA plate. The inoculant was allowed to soak into the agar and the plates were then incubated for 24 or up to 48 hr at 37º C. Resistance to an antibiotic was determined by comparing each isolate to the growth of that isolate on the control plate. A one (1) was recorded if that isolate grew to a round colony, and a zero (0) was recorded for no, or very little, growth. This was repeated for each isolate on each of the 28 antibiotic plates.

Table 3: Concentration and solvent of antibiotic stock solutions

Antibiotic:	Solvent:	Stock Conc. (mg/ml)
Oxytetracycline:	1:1 water methanol	10
Streptomycin:	distilled water	10
Cephalothin:	distilled water	10
Erythromycin	1:1 water ethanol	10
Tetracycline:	methanol	10
Neomycin:	distilled water	10
Rifampicin:	methanol	2.5

Table 4: Concentrations of antibiotic treatments

Antibiotic:	Plate concentrations (µg/l)
Oxytetracycline	2.5, 5.0, 7.5, 10, 15
Streptomycin	2.5, 5.0, 7.5, 10, 15
Cephalothin	15, 25, 35
Erythromycin	60, 70, 90, 100
Tetracycline	2.5, 5.0, 7.5, 10, 15
Neomycin	2.5, 5.0, 10
Rifampicin	60, 75, 90

C. Storage of Isolates of Known Origin

1. Freezing Known *Escherichia coli* cultures

A 10% solution of glycerol was used to freeze representative known source cultures. To make a 10% solution of glycerol, 90 ml of distilled water were put into a 250-ml flask that contained 10 ml of glycerol. Two-ml cryogenic vials were used to put

the known cultures containing the storage material. A small rack that holds 50 vials was used. With a pipette, 1.5-ml of 10% glycerol was added to each vial. The rack was labeled and then the vials were autoclaved to ensure sterilization. The vials were labeled by hand with the culture number and the date. Sterile technique was used during the inoculation of the vials with known cultures to ensure that the known cultures were not contaminated. The known *Escherichia coli* cultures were grown on EMB agar (Sigma) plates that had been inoculated with a metal replica-plater and incubated at 37º C overnight. Only the *Escherichia coli* cultures with a green metallic sheen were frozen. Sterilized loops were used to transfer colonies from the EMB plates to the sterilized vials. After inoculation, all the cultures were placed in the rack, which was then placed into the ultra-low freezer at -86º C. The cultures can remain in the rack on the shelf until it is convenient to place them in a labeled freezer box. The known cultures were then available to be used for future studies.

D. Statistical Analysis

The results from resistance testing of the known isolates were entered into the SAS statistical program (JMP Statistical Software, version 5.0) where they were analyzed by Logistic Regression using the Nominal Logistic procedure, which produces a classification table. The average rate of correct classification (ARCC) is the average rate that known isolates were correctly classified, and were determined by averaging the percentages of correctly classified isolates for each source. For this analysis, all resistance patterns from known sources were kept in the library. The isolates from each water sample were then classified using this library. Logistic Regression identifies the

most likely source for each isolate and displays the probability that each isolate belongs to the source that it is classified as.

When multiple regression methods are used to analyze relatively small data sets, random groupings (artificial clustering) based on stochastic processes rather than true relationships can occur. Such artificial classification limits the usefulness of small libraries, and should not exceed the purely random distribution of 20% for the five source categories used in this project. One-way to ensure that a library is large enough to avoid this random grouping phenomenon is to randomly assign the isolates to source categories as the library is being constructed. When analysis of the library is carried out, the ARCC for the randomly assigned data set should approximate the probability that an isolate would be assigned to a source category by chance.

To measure the representativeness of the library (i.e., how well it represents the diversity of patterns present in the sources in the watershed), all of the isolates from each known sample were successively removed from the library, and then classified based on the library containing the remaining isolates. The ARCC of these removed isolates was then calculated. This "jackknife" method estimates how well "new" isolates would be classified by the library. If there is a large difference between the ARCCs of these two methods, it suggests that the library is not representative of the sources in the watershed. The Minimum Detectable Percentage (MDP) (Sadowsky et al., 2000) for this library was calculated by determining the mean of the expected frequencies of misclassification (the average percentages of other source types that were misclassified as that type) and adding the value of 4 times the standard deviation of the mean (Whitlock *et al.,* 2002). This value is a conservative estimate of the minimum percentage of a source that can be

detected in a water sample. Thus, if a source is found at levels above the MDP, it can be reasonably assumed that this is not the result of misclassification of other sources, and therefore is present in the watershed.

Figure 1: Coan River Watershed

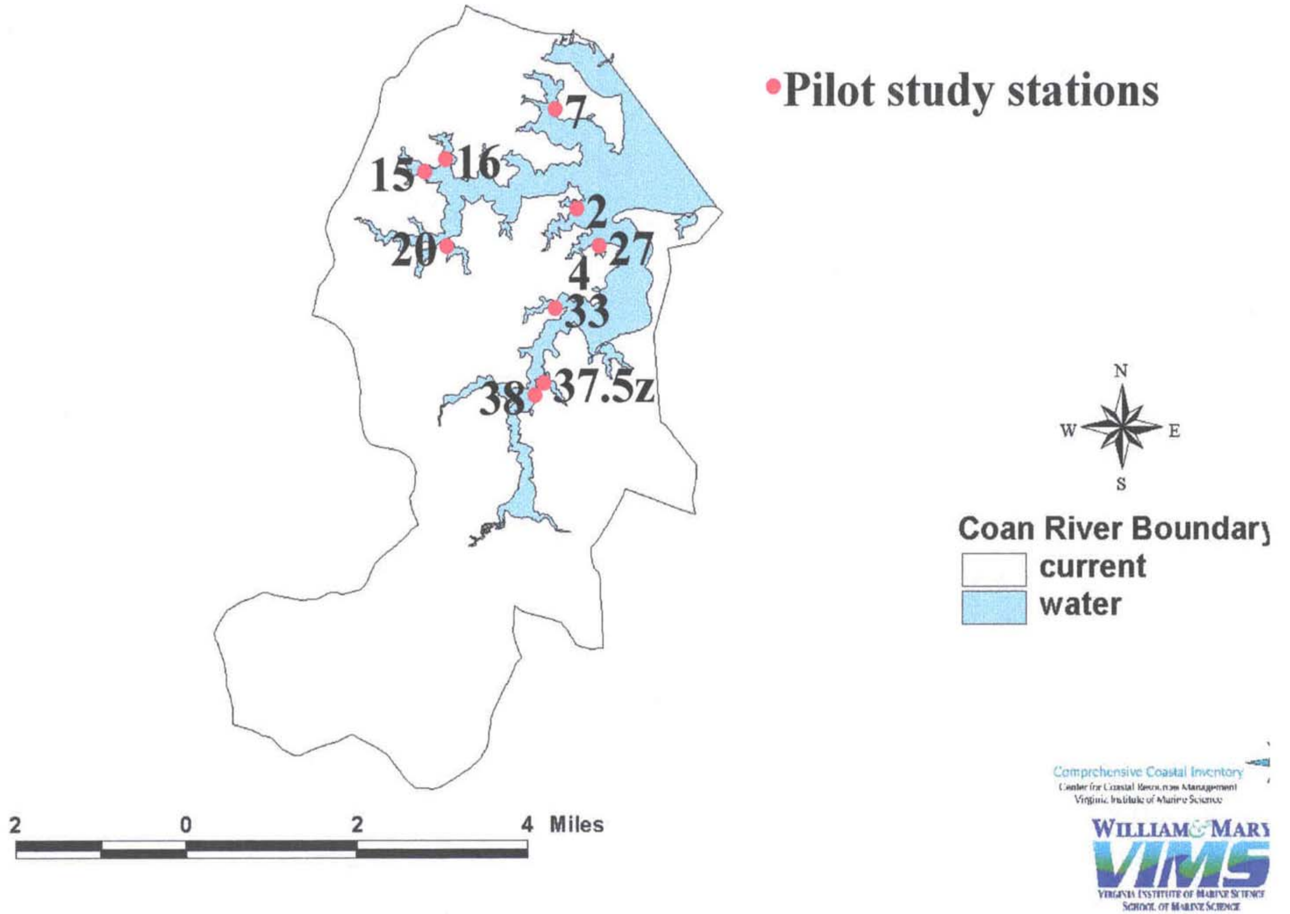

Figure 2: Little Wicomico River Watershed